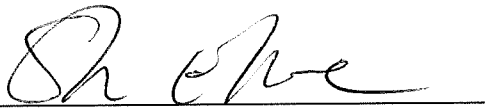
Exhibit 48

UNITED STATES DISTRICT COURT
DISTRICT OF NEW JERSEY

IN RE JOHNSON & JOHNSON (LHG) TALCUM POWDER PRODUCTS MARKETING, SALES PRACTICES, AND PRODUCTS LIABILITY LITIGATION ***THIS DOCUMENT RELATES TO ALL CASES***	**MDL NO. 16-2738 (FLW)**

RULE 26 EXPERT REPORT OF SARAH E. KANE, MD

Date: November 15, 2018



Sarah E. Kane, MD

I. BACKGROUND:

I am certified by the American Board of Pathology in Anatomic Pathology, Clinical Pathology and Cytopathology. I received my medical degree from The Ohio State University College of Medicine in Columbus, Ohio. I completed my residency in Anatomic and Clinical Pathology at Massachusetts General Hospital, a Harvard Medical School teaching hospital in Boston, Massachusetts. Following my residency, I completed a two-year gynecologic and cytology fellowship as the Robert E. Scully Fellow in Pathology at Massachusetts General Hospital, named after Dr. Robert Scully, who was a giant in the field of gynecologic pathology. This fellowship was focused on gynecologic pathology, perinatal pathology, and cytopathology. I studied the causes and mechanisms of disease as part of my training, and studied gynecologic cancer and disease in depth during my fellowship training. To this day, I routinely follow the gynecologic pathology literature as part of my regular practice.

I am currently a full partner in a private practice group, Commonwealth Pathology Partners PC. I have staff privileges at Massachusetts General Hospital, North Shore Medical Center (consisting of Salem Hospital in Salem, MA and Union Hospital in Lynn, MA) and Newton-Wellesley Hospital. I was hired by Commonwealth Pathology Partners PC to be the group's gynecologic pathology expert. Although all of the anatomic pathologists in our group practice general anatomic pathology, our group employs fellowship-trained pathologists in many subspecialty areas of pathology. This means that I see the majority of gynecologic surgical pathology specimens from my hospital sites, and if another pathologist needs an opinion on a gynecologic case, I will review it. I also presently serve as the autopsy director at North Shore Medical Center. I regularly attend and participate in numerous multidisciplinary conferences at Massachusetts General Hospital at the Cancer Center site in Danvers, MA.

Before entering private practice, I was a staff pathologist and Instructor of Pathology at Beth Israel Deaconess Medical Center (BIDMC), another Harvard Medical School teaching hospital. During my time at BIDMC, I performed specialty sign-out in gynecologic pathology, perinatal pathology and cytology. I was also served as the Associate Director of the Cytopathology Fellowship Program at BIDMC, served on numerous pathology department committees, and taught several courses at Harvard Medical School before I was recruited for my current position. My curriculum vitae is attached as Exhibit A. It further details these positions and the remainder of my work experience in this field. Exhibit B details the references cited in this report, as well as other materials and data I considered.

I have been asked to provide an expert report regarding my opinions on the question of general causality in the case of talcum powder product use and ovarian cancer. All of my opinions stated below are held to a reasonable degree of medical and scientific certainty. I reserve the right to modify or change my opinion based on further documents or information that may be provided to me in the future.

A pathologist is a physician who has completed medical school and a post-graduate residency in pathology (either clinical pathology, anatomic pathology, or both).Like me, many pathologists go on to complete fellowships following their education and residency.

Pathology is the study of disease; pathologists spend much of their time both in training and in daily practice studying the causes and presentations of disease. The years of medical training are of critical importance in daily practice; pathologists must make clinical assessments, based in part on medical and epidemiologic knowledge, about identification of causes, risk factors, clinical sequelae, morphologic, and genetic features of disease.

In order to produce accurate diagnoses, pathologists must be knowledgeable about the medical, scientific, and epidemiologic evidence base. A knowledge of advancements in technologies applied to tissue samples must be continuously maintained. This involves not only maintaining current knowledge of the pathology literature, but also of the literature in various other fields such as oncology and other fields relevant to our practice.

One of the tools used in the process of identifying talc particles in tissue is polarized light microscopy. Anatomic pathologists routinely use polarized light microscopy in clinical practice. As an example, one might use polarized light microscopy to find foreign material and explain an inflammatory reaction. The most common application in my practice is for identifying calcium oxalate crystals in breast biopsies done for radiologically identified calcifications. I estimate I use polarized light microscopy for this purpose about twice a month.

In anatomic pathology, the pathologist not only needs to be aware of the numerous possible diagnoses, but also of the causes of diseases one may encounter in any given organ system. Coming to a diagnosis requires knowledge of the medical, scientific, and epidemiologic literature. Pathologists must be proficient in the current literature that informs and supports their conclusions.

Ultimately, a pathologist's diagnosis must make biological sense and must be supported by the weight of the available medical and scientific information. Not only must a particular case match the morphological characteristics of the diagnosis being made, but it must fit the clinical presentation, the patient history, and it must be consistent with what is known about the disease, including what is known about disease causation. These are the same medical and scientific information resources that I rely on for my opinions in this report.

Thus, the work that I've done in this report is similar to what I do in my daily practice. My clinical practice requires ongoing familiarity with the same medical evidence that I have considered here.

Ovarian cancer has an incidence rate of 11.8 per 100,000, and thus is relatively rare (Torre 2018). At my current private practice, I am the primary pathologist on approximately 6,000 cases annually. This includes both surgical pathology and cytopathology cases. I would be diagnosing, ruling out, or looking for ovarian cancer or metastatic ovarian cancer (among other diseases), in approximately 2000 cases a year as a rough estimate. Of those, I estimate that I diagnose about 30 cases per year as ovarian tumors. Academic teaching hospitals generally tend to have a higher volume of ovarian tumor cases due to their large referral bases. While I was a staff pathologist at Beth Israel Deaconess Medical Center, the pathology department implemented a subspecialty sign-out schedule in 2010. In my last two years there,

I signed out predominantly gynecologic surgical pathology in addition to cytopathology (in prior years the department had a general surgical pathology schedule, which meant all types of cases went to each anatomic pathologist regardless of subspecialty fellowship training). During that time, I estimate I signed out about 500 ovarian tumor cases per year. Similarly, while I was a fellow at Massachusetts General Hospital from 2005-2007, I independently signed out gynecologic surgical pathology and estimate I signed out approximately 500 ovarian tumor cases per year. As a resident in anatomic pathology at Massachusetts General Hospital, I was exposed to hundreds of ovarian tumor cases both during my clinical case work and didactic sessions.

Of note, during my time at Massachusetts General Hospital, both Drs. Robert Scully and Debra Bell were still working in the Department of Pathology. Dr. Scully was a co-author on Dr. Cramer's first paper on talc and ovarian cancer in 1982, and Dr. Bell was a co-author on Drs. Harlow and Cramer's 1992 paper on talc and ovarian cancer. Dr. Bell's tenure as Cytopathology Director also overlapped with my time there. This meant that I spent significant time with Dr. Bell during my residency and fellowship. I was the primary author of a paper on ovarian serous borderline tumors in 2006, with Dr. Bell serving as a co-author. Dr. Scully, known as a giant in gynecologic pathology, was semi-retired by the time I started my pathology residency in 2001. However, he was at the hospital nearly every day and all of the gynecologic pathologists would still show him cases on a consult basis. Dr. Robert Young, the director of my fellowship program, was a Scully protege and continued his consulting practice. It is because of my training at Massachusetts General Hospital and my interactions with both Drs. Scully and Bell that I first became aware of their work on talc and ovarian cancer. Since then, I have maintained a professional interest in and have continued to monitor developments in the science regarding talcum powder exposure and ovarian cancer, and it has been the subject of professional discussions pre-dating this litigation.

My billing rate is $500 per hour. I have previously testified in one matter, a deposition for the case of Julie Lagadimas, as Personal Rep. of the Estate of Dawn M. O'Toole v. R.J. Reynolds Tobacco Co., et al; Norfolk Super. Ct. Case No. 1582-CV-01474.

II. GENERAL CAUSATION OPINIONS:

Based on assessing and weighing the totality of the evidence, and following the methodology set forth below, I hold the following opinions to a reasonable degree of scientific and medical certainty:

1. Talcum powder products and their constituent minerals can reach the ovaries through migration up the genital tract from the perineum to the fallopian tubes and ovaries. There is also evidence that these products can be transported through the lymphatic system (Cramer 2007). Another biologically plausible pathway is inhalation leading to lymphatic transport to the ovaries (Suzuki 1991, Marchiori 2010, Frank 2011).

2. Once reaching the ovaries, talcum powder products can cause chronic inflammation, can increase oxidative stress, and can reduce immune response. These are biologically plausible and likely mechanisms for ovarian cancer development and progression.

3. There are chemical similarities between asbestos and talc and there are striking pathological similarities between invasive serous ovarian cancer and mesothelioma.

4. There is evidence that talcum powder products manufactured by Johnson & Johnson (Johnson's Baby Powder and Shower to Shower) have contained and continue to contain asbestos, talc containing asbestiform fibers (fibrous talc), and heavy metals such as cobalt, nickel, and chromium. Other than cobalt, which has been identified as a "possible" carcinogen by the International Agency for Research on Cancer (IARC), all of these constituents have been identified as known carcinogens by IARC (IARC 1987, IARC 2012).

5. For purposes of my opinions, I have reviewed and relied upon Dr. Crowley's report regarding the fragrance chemical constituents in Johnson & Johnson talcum powder products (Crowley Report), as well as testing reports and analysis which include, Dr. Blount (Blount Report), Dr. Longo and Dr. Mark Rigler (Longo et al. Report), as well as the corporate testimonies of John Hopkins and Julie Pier. The presence of these constituents as part of talcum powder products provides additional evidence of biological plausibility for causation regarding talc and ovarian cancer.

My opinions and conclusions are supported by epidemiologic studies showing an increased risk of ovarian cancer in women who used talcum powder products for perineal dusting, animal and in vitro studies, cellular biology studies, and pathological evidence which provides a highly biologically plausible mechanism for talc's carcinogenicity. Based on the totality of evidence, it is my opinion to a reasonable degree of scientific and medical certainty, that perineal exposure to talcum powder products can cause epithelial ovarian cancer.

III. METHODOLOGY FOR ASSESSING CAUSATION AND PRINCIPLES OF CAUSAL INFERENCE:

For this report, I followed the same methodology that I use in my clinical practice and research, a method that is generally accepted in the medical community. I used the same standards for evaluating and interpreting medical and scientific evidence, and I followed generally accepted standards in science and medicine for assessing causation, including consideration of the Bradford Hill viewpoints.

My causal assessment in this case is based on my background, training, education and experience as a physician and pathologist in interpreting, comparing, and weighing the totality of the available biologic, pathologic and epidemiologic evidence. I considered this evidence in the context of the Bradford Hill causation assessment viewpoints to reach an opinion regarding whether talcum powder products[1] can cause epithelial ovarian cancer.

Bradford Hill's discussion of a causal relationship includes strength of association, consistency, coherence, specificity, temporality, biological plausibility, dose-response, experimental evidence, and analogy as different "viewpoints" of a causal relationship between

[1] In my report, the term "talc" is used to refer to talcum powder products.

an exposure and a disease. Consideration of Bradford Hill's approach to causation, which I discuss in more detail below, supports general causation of talcum powder product exposure and ovarian cancer. The Bradford Hill causation viewpoints are not a checklist of requirements, and it does not call for a mechanical application of his 9 considerations for assessing a causal relationship; rather, it is properly understood as providing a framework for an assessment of the totality of the evidence leading to a judgment about causation. As Bradford Hill himself put it, "What I do not believe…is that we can usefully lay down some hard-and-fast rule of evidence that must be obeyed….None of my nine viewpoints can bring indisputable evidence for or against the cause-and-effect hypothesis and none can be required as the *sine qua non*." I agree with that statement.

My methodology began with a systematic review of the medical literature to ascertain the relevant body of scientific evidence that I would consider. This included consideration of a large number of peer-reviewed publications reporting the results of human epidemiological studies investigating the association between talc exposure and ovarian cancer. I also considered and weighed other lines of evidence pertaining to explaining relevant, plausible, and likely mechanisms for how talcum powder product exposure causes ovarian cancer. This included carcinogenicity studies and data regarding talc and its constituents. Counsel for plaintiffs also provided me with medical literature to review, most of which overlapped with materials that I found independently through my own medical literature searches.

Relevance is not simply a yes/no proposition; it is a variable that ranges from not relevant to directly relevant, and there is a range between these extremes. Only a careful review of the evidence leads to an assessment of the degree of relevance. Much of science involves extrapolation and generalization from one study to the general population. The assessment of relevance is based on the extent that the study results are pertinent to the issue under consideration.

Human data is generally more relevant than animal data when assessing causation in humans. However, animal studies on exposure and disease are performed to advance our understanding of the human response to the same dose-adjusted exposure, and thus animal data is often relevant and important in that it can provide important information that forms part of the total evidence assessment. For example, if an exposure to talc in a rat causes inflammation, that could be relevant to assessing the effect in humans.

All observational studies have limitations, requiring careful interpretation. Reliability determinations focus on the degree of confidence in a study's internal validity. Reliability, like relevance, is not a yes/no proposition. For human epidemiologic observational studies, reliability assessments entail consideration of alternative explanations, including the role of chance and the likelihood that the results are affected by bias or confounding. Factors to be considered include: (1) Do we have reliable and appropriate measures of exposure; (2) do we have reliable assessments of disease; (3) do we have comparable groups for comparison; (4) have the investigators adjusted for potential confounding; (5) are the study results likely the result of a systematic bias; and, (6) does the study have enough exposures and sufficient power to detect an association if it exists?

I also consider the type of study design and whether it is suited to the question being researched. There is a general hierarchy of evidence, which I also consider, but study type and its position in the hierarchy will only have value if the study is otherwise relevant and reliable. For example, a randomized clinical trial may be the "gold standard," but one must still look at whether the study does in fact provide a relevant and reliable result for the issue of interest (here, whether talcum powder products are capable of causing ovarian cancer).

In weighing the evidence other important considerations include: How does the study define, ascertain, and measure talc exposure? What type of study was it? Other considerations include: Has the study been or can it be replicated? Is the study result consistent with other studies? Has the study been published and has it been peer reviewed? Has the study been conducted on a relevant population? How does the study adjust for potential confounders and how does the study minimize or account for bias? Is there a potential for misclassification of exposure or disease based on the circumstances under which the data was gathered or analyzed? What is the potential that study results could be due to chance, bias, or confounding? Is there a statistical analysis, with a reported error rate? Were the results statistically significant, and, if not, are the results still important when considered with all other evidence from the perspective of overall consistency? What is the size of the study population? Is the study large enough to detect an association if it exists? Do the results make biologic sense? This is a list of examples of considerations for weighing the evidence, and is not intended to be comprehensive.

In weighing the evidence, I also consider the reported "P values" and confidence intervals (the result of statistical calculations), along with the reported relative risks and odds ratios, and other details about each study as explained above and below. The concept of "statistical significance" is often misunderstood. In assessing any statistical evidence pertaining to medical issues, medical and scientific researchers note whether certain findings are "statistically significant." However, findings that are not "statistically significant" are often statistically and clinically important and should be considered and weighed along with other available evidence in making causal assessments. The concept of statistical significance using arbitrary cutoffs has no relationship to the strength or direction of an estimated association, and may have very little relationship with the actual validity of a study's results. A "P value" of 0.05 or less is often considered statistically significant, whereas 0.06 is not.[2] I agree with the epidemiologists who consider this "cut-off" to be arbitrary, because, for example, the .01 difference between p = 0.05 and p. = 0.06 is essentially the difference between a 5% vs. 6% probability that the observed association is due to the role of chance. Even where a confidence interval includes "1," depending on the values of the lower and upper bounds of the confidence interval, the most likely interpretation of the study results may be that there is an association between an exposure and the increased risk of a disease.

[2] In epidemiologic studies, epidemiologists or statisticians calculate a P-value and/or 95% confidence interval ("CI") for each risk estimate. Essentially, the P-value and the CI assess the likelihood that the observed association is due to the play of chance. A 95% CI means that if the same experiment is repeated many times, 95% of the time, the true value of the risk estimate will fall between the upper and lower bound of the CI. The narrower the CI, the more precise and reliable the risk estimate is considered to be.

Bradford Hill stated that "[n]o formal tests of significance can answer those questions [of causation]. Such tests can, and should, remind us of the effects of the play of chance… Beyond that, they contribute nothing…" Therefore, in weighing the evidence, I note the P-value and/or the confidence interval reported with a study's results, and consider this to be an important piece of information for interpreting study results. I do not think it is appropriate to disregard results just because they do not meet an arbitrary statistical threshold, a view also held by the American Statistical Association (Wasserstein 2016).

All observational studies have limitations, and the potential for "bias" and confounding. The presence of some bias is not generally a basis for scientists to disregard a study. Instead, when interpreting a study, biases must be considered and assessed for the likelihood that they may obscure, diminish, or magnify a study result, so the direction and magnitude of any bias must also be considered where possible. Some biases will have the effect of obscuring or understating an association between exposure and disease. Typically, study investigators will include as part of their published paper reporting the study results, the important strengths and limitations (including their assessment of the role of bias, chance and confounding) in the study.

In weighing the evidence, I also consider the likelihood that the study may understate or fail to detect an association that did exist (a Type II error, often due to lack of "power"); or the converse, that a study result may overstate an association or find an association that is not real (Type 1 error). In interpreting studies that do not report an association with an increased risk of ovarian cancer, one issue is whether the results provide reliable evidence of the absence of an association. The only way for data to provide statistical reassurance about the absence of an association is, in the absence of any important systematic error in the data, for the upper bound of a reasonable confidence interval (such as a 95% confidence interval) to be close to the null value.

When a study finds an association between exposure and disease, causation is one explanation, but it is not the only explanation. Other explanations must be considered and assessed. When an observational study results in a reported association between exposure and disease (i.e., relative risk or odds ratio greater than 1.0), and if alternative explanations (*i.e.,* the role of bias, confounding and chance) are considered and determined to be unlikely explanations, then causation remains a likely explanation, subject to consideration of the Hill viewpoints. In order to reach an opinion that an association is causal between talc exposure and ovarian cancer, I considered whether there are other potential explanations that better explain the relationship and which are consistent with the totality of the scientific evidence. This assessment is informed by considering how a specific study fits into the overall totality of the evidence.

My opinions on causation are informed by a review of the strengths and limitations of the epidemiology evidence along with a review of other lines of evidence, including animal data and evidence on biological plausibility, likely mechanism(s) and dose/response. Thus, as part of my methodology, I have considered whether there is an alternative explanation to causation, based on an assessment of the totality of evidence. For example, I have considered whether the findings of the human epidemiologic studies are best explained by chance,

confounding or bias, when viewed separately, and most importantly, when viewed as a whole, and in light of the several lines of experimental evidence discussed in this report.

Based on my review of the totality of evidence, which I have weighed based on the considerations described above, I conclude with a high degree of medical and scientific certainty that exposure to talcum powder products can cause ovarian cancer. Causation is the best explanation for assimilating, assessing and weighing the totality of evidence. In reaching this opinion, I found it compelling that the epidemiologic studies that captured talc exposure consistently found an association between exposure to talc applied in the perineal area and epithelial ovarian cancer. The studies also provide persuasive evidence of a dose response effect, one of the viewpoints of causality discussed by Bradford Hill. There also is persuasive evidence of plausible and likely causal mechanisms for how talc exposure leads to ovarian cancer.

The other explanations for an association (other than causation) are bias, chance and confounding, and "reverse causation."[3] While it may not possible when looking at a single study to determine whether a recall bias, or a selection bias, or a potential confounder is materially affecting the results, I find it helpful to consider how each study fits into the whole. Here, multiple studies have been conducted in different populations, by different investigators, using different methods, and using different study types, and yet there is general consistency in the results. The vast majority of studies and meta-analyses find an association with an increased risk of ovarian cancer. Under these circumstances, viewing the evidence as a whole, the likelihood that the consistent finding of an association can be explained by bias, or chance or confounding is highly unlikely, especially in light of the results of the other lines of evidence.

Finally, as part of my methodology of considering alternative explanations for the evidence, I made an effort to understand the opinions of both the plaintiff and defense experts as concerning the issue of talc and causation of ovarian cancer. In that regard I have reviewed some plaintiff and defense expert testimony and reports, which are identified on my reference list. I also cited to the extensive medical literature I considered in connection with my work on this report.

IV. MECHANISM OF TALC'S CARCINOGENICITY

There is a plausible and likely biologic mechanism whereby talc causes inflammation which can lead to epithelial ovarian cancer. Chronic inflammation has been causally linked to a number of cancers. The evidence of the relationship between inflammation and cancer is based on many studies, including studies supporting the

[3] In epidemiology, reverse causation is when the exposure-disease process is reversed; In other words, the exposure causes the risk factor. Here, the question is whether exposure to talcum powder products causes ovarian cancer or whether ovarian cancer causes increased usage of talcum powder products? I am not aware of any evidence to support a conclusion that reverse causation is a plausible explanation for the association between exposure to talcum powder products and ovarian cancer. The principal presenting symptom is abdominal bloating, which does not appear to lead to more talc use.

conclusion that inflammation plays a role in increasing the risk of epithelial ovarian carcinoma. As stated by the National Cancer Institute, "Over time, chronic inflammation can cause DNA damage and lead to cancer. For example, people with chronic inflammatory bowel diseases…have an increased risk of colon cancer." The time interval between inflammatory response and presentation of cancer can be many years. Animal studies, particularly, may show granulomatous or other inflammatory reactions while not necessarily demonstrating neoplastic changes due to the time interval required for cancer to develop.

Studies have shown that pelvic inflammatory disease and endometriosis (known to cause an inflammatory reaction) increase the risk of ovarian cancer (Risch 1995, Brinton 1997, Ness 2000, Brinton 2004, Kobayashi 2007, Lin 2011, Zhou 2017). Genofre et al. (2007) showed that talc can induce inflammation. Ness (1999) reported that inflammation of ovarian epithelium is a risk factor for ovarian cancer.

Inflammation has been implicated in carcinogenesis in several ways. Inflammation increases cytokines (Ness 1999). Shukla (2009) showed that nonfibrous talc can induce an inflammatory response that alters expression of genes in cancer pathways such as COX-2, ATF3, IL-6, and IL-8 in mesothelial cells. Further, inflammation increases oxidative stress (Ness 1999); Buz'Zard (2007) revealed that talc can induce oxidative stress and create reactive oxygen species (ROS), which in turn can induce ovarian neoplastic transformation in human ovarian cells. See also Saed (2017).

V. INFLAMMATION

Inflammation can produce toxic oxidants such as ROS that can be a source of mutagenesis to DNA. This damage to DNA by ROS is now accepted as a major cause of cancer, and has been demonstrated in ovarian cancer (Senthil 2004, Saed 2010, Saed 2017) as well as in breast and hepatocellular carcinoma (Waris 2006, Saed 2017). Talc exposure has been shown to cause a statistically significant increase in ROS in ovarian polymorphonuclear neutrophils (PMNs), resulting in a decrease in cell viability and neoplastic transformation of ovarian cells. The authors concluded that "talc increased proliferation, induced neoplastic transformation and increased ROS generation time-dependently in the ovarian cells." (Buz'Zard 2007)

Thus, it is accepted that inflammation causes oxidative stress. Oxidative stress leads to the formation of ROS and reactive nitrogen species (RNS). Oxidative stress is an important factor in the initiation and development of several cancers, including ovarian cancer (Senthil 2004, Saed 2010, Saed 2018). The production of oxidants and free radicals affects cellular mechanisms that control cell proliferation and apoptosis, which in turn play a role in the initiation and development of several cancers (Saed 2018). ROS and RNS can induce genetic mutations and DNA damage, thus causing oncogenic phenotypes. Additionally, oxidative stress affects transcription factors that modulate the expression of genes important to the development and metastasis of cancer cells (Saed 2018). Oxidative stress is also known to activate certain signaling pathways, which are critical for the initiation and maintenance of the oncogenic phenotype (Waris 2006). In fact, the major source of cellular ROS, the NAD(P)H

oxidase family of enzymes, has been linked to the survival and growth of tumor cells in pancreatic and lung cancers (Reuter 2010, Rojas 2016). Pro-oxidant enzymes such as myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS), and NAD(P)H oxidase have been associated with initiation, progression, survival, and increased risk in cancers such as breast, ovarian, lung, prostate, bladder, colorectal, and melanoma (Lengyel 2010, Fletcher 2017, Saed 2017, Saed 2018). Angiogenesis is critical for the survival of solid tumors and is also regulated by ROS (Reuter 2010, Saed 2017). Thus, it is clear that alteration of oxidative balance can provide an environment for cancer cell survival (Saed 2018).

Gene point mutations resulting in single nucleotide polymorphisms (SNPs), or a variation in a single base pair in DNA, have been associated with oxidative DNA repair genes and redox genes with cancer susceptibility (Klaunig 2010). There is evidence that genetic polymorphisms in genes with anti-tumor activity are associated with cell cycle genes and play a role in ovarian cancer etiology (Goode 2009, Notaridou 2011). There are associations of specific SNPs in oxidant and anti-oxidant enzymes with increased risk and survival of ovarian cancer (Belotte 2015, Fletcher 2017).

Higher levels of oxidants have been described in epithelial ovarian cancer (Malone 2006, Saed 2010, Jiang 2011). Fletcher et al. published an abstract in the March 2018 Reproductive Sciences that showed talc can generate a pro-oxidant state in both normal ovarian epithelial and ovarian cancer cells. In this study, there was a marked increase in mRNA levels of the pro-oxidant enzymes iNOS and MPO in talc treated ovarian cancer cell lines and normal ovarian epithelial cells, as compared to controls within 24 hours. There was also a marked decrease in the mRNA levels of the anti-oxidant enzymes catalase (CAT), glutathione peroxidase (GPX), and superoxide dismutase 3 (SOD3), but a marked increase in glutathione reductase (GSR) and no change in glutathione S-transferase (GST) in the talc treated ovarian cancer cell line and in normal ovarian epithelial cells compared to controls within 24 hours (Fletcher 2018). In addition to tumorigenic cells generating high levels of ROS that activate signaling pathways which promote proliferation, it is known that tumorigenic cells maintain a high level of antioxidant activity to prevent buildup of ROS to levels that could induce tumor cell death (Schieber 2014, Saed 2017).

ROS and RNS are normally neutralized by enzymes such as SOD, CAT, GST, glutathione (GSH), thioredoxin coupled with thioredoxin reductase, glutaredoxin, glutathione peroxidase (GPX), and GSR (Lei 2016). Glutathione S-transferase is involved in detoxification of carcinogens by catalyzing their conjugation to GSH (Lei 2016). The GS-X-MRP1 efflux pump, which removes toxins from cells, is known to be stimulated by the GSH/GSSG complex and this process has been investigated as a mechanism for the development of tumor chemoresistance (Ishikawa 1993, Circu 2012).

Further, data demonstrates that talc exposure caused a statistically significant increase in ROS in ovarian polymorphonuclear neutrophils (PMNs), which resulted in a decrease in cell viability and neoplastic transformation of ovarian cells (Buz'Zard 2007).

Additionally, inflammation induces increased cellular proliferation, giving rise to potential DNA replication errors. This is one of the ways increased lifetime ovulations increase the risk of epithelial ovarian carcinomas. Studies have shown that ovulation results in an inflammatory response to disruption of the ovarian epithelium with the release of inflammatory mediators that initiate cellular transformation and growth (Richards 2002). Endometriosis causes an inflammatory reaction (including macrophage activation, cytokine release, and expression of growth factors) and is a risk factor for clear cell (Figure 4) and endometrioid (Figure 5) ovarian carcinomas (Risch 1995, Brinton 1997, Ness 2000, Brinton 2004, Kobayashi 2007, Edwards 2015). Studies have also shown that pelvic inflammatory disease (PID) is an ovarian cancer risk factor (Risch 1995, Brinton 1997, Ness 2000, Brinton 2004, Kobayashi 2007, Lin 2011, Zhou 2017). Several prospective studies suggest that elevated serum levels of inflammatory markers such as CRP, TNF-α and IL-6 are predictive of development of ovarian cancer (McSorley 2007, Lundin 2009, Clendenen 2011, Toriola 2011, Poole 2013, Trabert 2014, Gupta 2016).

There also are some studies showing a protective effect of anti-inflammatory drugs on the risk of developing carcinoma, although some studies have failed to show a protective effect (Wu 2009). An analysis of many randomized controlled studies did show a reduced risk of developing carcinoma with aspirin use (Rothwell 2012). A 2014 article specifically evaluating ovarian carcinoma analyzed pooled data from 12 population-based case-control studies and showed a reduction of ovarian cancer risk with frequent aspirin and high-dose non-steroidal anti-inflammatory (NSAID) use (Trabert 2014). This further supports the role of inflammation in carcinogenesis, as this effect cannot be explained by other etiologies (Baandrup 2013, Trabert 2014).

Talc is not an inert substance. It has been shown to cause inflammation. Studies have shown increases in markers of inflammation following talc exposure (Allaire 1989, Genofre 2007, Arellano-Orden 2013). Talc is used therapeutically for patients with recurrent pneumothorax and pleural effusions based upon its ability to induce inflammation and adhesions. Injecting talc into the pleural space causes an inflammatory and granulomatous reaction, causing fibrosis and scarring which prevents further pneumothorax development (Antonangelo 2006, Najmunnisa 2007). This is mediated through the release of cytokines and chemokines (Nasreen 1998, van den Heuvel 1998), and the production of basic fibroblast growth factor (bFGF) (Antony 2004). It is worth noting that asbestos fibers are also known to initiate an inflammatory and scarring process within the pleura and peritoneum, which can eventually lead to neoplastic transformation of the mesothelium. The time interval between the initial inflammatory response for asbestos and talc and the development of cancer can be many years. Remote exposure will not necessarily mean there will be evidence of current inflammation or foreign body reaction when tissues are examined.

There also is evidence that talc induces macrophage TNF-α expression (Cheng 2000). Macrophages that express TNF-α promote ovarian tumorigenesis (Hagemann 2006). TNF-α is involved in chronic inflammation and induces mutations in vitro (Yan 2006). TNF-α induced chromosomal mutations occur mostly in cells with p53 aberrations (Yan 2006). Of note, high grade serous carcinomas typically have inactivating mutations in p53. Both talc and TNF-α induce macrophage expression of IL-8 (Nasreen 1998, van den Heuvel 1998), which attracts

neutrophils that then release ROS. This in turn causes a feedback loop between ROS generation and increased TNF-α expression, causing increased DNA damage (Xie 2000). This is an important line of biological experimental evidence supporting my causation opinion. The strongest association of talc and ovarian cancer is with invasive serous carcinomas, which commonly have p53 mutations, and TNF-α induced chromosomal mutations occur mostly in cells with p53 aberrations. Talc has been shown to induce macrophage TNF-α expression, which has been shown to promote ovarian tumorigenesis.

VI. ROLE OF IMMUNE SYSTEM IN CARCINOGENESIS

Studies have evaluated the protective role of the immune system in carcinogenesis, and in particular anti-MUC1 antibodies (Cramer 2005). MUC1 is a high molecular weight transmembrane protein expressed in many normal organs in a highly-glycosylated form. In cancer, including ovarian carcinoma, MUC1 is expressed at high levels in a poorly-glycosylated form. Anti-MUC1 antibodies are produced when high levels of the poorly-glycosylated form of MUC1 present to the immune system. Anti-MUC1 antibodies have been found in some cancers (Ho 1993, Dong 1997, Feng 2002) and have been associated with improved prognoses (Kotera 1994). Chronic processes including endometriosis, ovulation and talc exposure affect expression of MUC1 (Cramer 2005, Vlad 2006, Terry 2007). Decreased anti-MUC1 antibody production caused by these processes plausibly leads to immune-tolerance of an early ovarian carcinoma. Cramer et al. published a paper in 2005 that showed factors which increase the levels of anti-MUC1 antibodies lower the risk of ovarian carcinoma (Cramer 2005). Factors that decrease anti-MUC1 antibodies, such as incessant ovulation, have been associated with an increased risk of ovarian carcinoma (Terry 2007). Prospective data from the Nurses' Health Study (NHS) showed that tubal ligation increases anti-MUC1 antibodies, potentially by the procedure triggering the production of anti-MUC1, thus indicating another way tubal ligation exerts its protective effect. The study also showed that increased numbers of ovulatory cycles decrease anti-MUC1 antibodies, providing an explanation for the increased risk of ovarian cancer with increased lifetime ovulations (Pinheiro 2010). These studies provide evidence that MUC1 antibodies serve a role in the mechanism of and immune response in ovarian carcinogenesis. Because talc use is associated with a decrease in MUC1 antibody expression, the above is relevant to assessing the risk of talc use and ovarian cancer and provides further evidence supporting causation.

VII. COSMETIC TALC

Cosmetic talc has been used for decades, applied directly or indirectly to the genital region because of its high absorbency and softness (Langseth 2008).

Talc is a magnesium silicate hydroxide, characterized by water molecules in between silicate sheets. Asbestos is also a silicate mineral, but is somewhat morphologically distinct from talc and belongs to different silicate mineral groups. However, the chemical similarity of asbestos and talc led some researchers to postulate that both talc and asbestos could be causes of ovarian cancer (Graham 1967, Henderson 1971, Longo 1979). Early research into the possible link between talc and ovarian cancer was also instigated due to the fact that high

grade serous carcinoma, a type of invasive serous epithelial ovarian cancer (Figure 1), shown to be most commonly associated with perineal talc use, has striking morphologic similarities to mesothelioma (Figure 2), the tumor most associated with asbestos (Graham 1967). High grade ovarian serous carcinoma and mesothelioma express similar immunohistochemical markers, most notably cytokeratin pattern, WT-1 and calretinin. In fact, a great deal of surgical pathology literature deals with the nuances in differentiating peritoneal mesothelioma from high grade serous carcinoma. In the last few years, additional immunohistochemical panels have been developed that help distinguish between these two tumors (Laury 2010, Ordonez 2013), including PAX8, which is also expressed in fallopian tube epithelium. The morphologic and immunohistochemical similarities between asbestos and talc malignancies constitute another line of evidence supporting my opinion that talc exposure in the genital area causes ovarian cancer. Later in this report, I address the evidence that asbestos exposure can cause ovarian cancer.

VIII. TALC MIGRATION, TRANSLOCATION, INHALATION, AND LYMPHATIC TRANSPORT

In order for cosmetic talc applied to the perineum to reach the ovary or fallopian tube and exert a neoplastic effect, it needs to travel up through the vagina and uterus. It is known that substances can travel proximally through the female genital tract to the fallopian tubes and ovaries (Egli 1961, Venter 1979). Several studies have demonstrated the presence of talc in ovarian tissue (Henderson 1971, Henderson 1979, Mostafa 1985, Heller 1996) and even in the pelvic lymph nodes of a woman with ovarian cancer and long-term genital exposure to cosmetic talc (Cramer 2007). This is evidence that talc can be transported through the lymphatic system. Thus, another biologically plausible pathway is inhalation leading to lymphatic transport to the ovaries (Suzuki 1991, Marchiori 2010, Frank 2011).

There is evidence that serous ovarian cancers are actually of fallopian tube origin (Piek 2003, Kindelberger 2007, Kurman 2010, Erickson 2013). When considering whether talcum powder can cause ovarian cancer, this consideration is not critical. Talcum powder particulates reach both the fallopian tubes and ovarian surfaces by migrating proximally.

IX. TALC IN TISSUE

As mentioned above, several studies have demonstrated the presence of talc in ovarian tissue (Henderson 1971, Henderson 1979, Mostafa 1985, Heller 1996) and one study found talc in the pelvic lymph nodes of a woman with ovarian cancer and long-term genital exposure to cosmetic talc (Cramer 2007). In Cramer et al.'s 2007 paper, the methods used by Dr. John Godleski to identify talc particles in tissue are outlined (Cramer 2007).

Tissue was first analyzed using polarized light microscopy to identify birefringent particles within the tissue plane. Polarized light microscopy is used in routine practice in anatomic pathology. One of the most common uses in surgical pathology is for the identification of calcium oxalate calcifications in breast tissue. In some lesions of the breast,

ranging from benign to malignant, calcifications occur that can be a marker for disease and are discovered on breast mammography. After mammography reveals calcifications and the radiologist determines them to be suspicious for disease, the area with calcifications is biopsied. The biopsy sample is then X-rayed to confirm the presence of the calcifications, and then submitted to the pathology laboratory for histologic analysis and diagnosis. The pathologist correlates the calcifications seen under the microscope with those in the specimen X-ray to be sure the calcifications the radiologist identified are visualized in the tissue sample. Calcium oxalate is a certain type of calcification that is not easily seen on light microscopy. If there appears to be a discrepancy between the tissue under light microscopy and the specimen X-ray (lack of calcifications under light microscopy), the pathologist will use polarized light microscopy to help identify calcium oxalate crystals, which are birefringent. Similarly, Dr. Godleski used polarized light microscopy to identify birefringent material that could be further analyzed using SEM and EDX.

SEM was more commonly used in surgical pathology before immunohistochemical studies were routinely used and before the common availability of molecular testing. However, SEM is still routinely used as an important diagnostic tool in areas of pathology in which immunohistochemical studies and molecular testing are less helpful, such as medical renal pathology, neuromuscular disorders and rare tumors. SEM uses electrons for imaging, analogous to light microscopy using light. SEM allows for much greater magnification (>100,000X) than light microscopy.

EDX is a qualitative and quantitative chemical analysis used in conjunction with SEM. It detects X-rays emitted from the sample during electron scanning to determine the elemental composition of the particle being examined. EDX is widely used in many biomedical areas, as it provides precise information on the chemical composition of subcellular structures that can be correlated with their SEM images (Wyroba 2015).

In Cramer et al 2007, the authors analyzed four pelvic lymph nodes from a 68 year old woman with ovarian papillary serous carcinoma and a small component of clear cell carcinoma. She had been a daily talc user for 30 years, having applied it to underwear and sanitary napkins. The lymph nodes showed birefringent particles via polarized light microscopy and were then analyzed by SEM and EDX. This showed magnesium and silicate signatures consistent with talc (Cramer 2007). Of note, there are similar studies performed with asbestos fibers in tissue sections (Roggli 1983, 1986).

Additionally, studies have shown Raman microscopy can be used to identify talc spectra in routinely processed, but unstained, histologic pathology specimens. Raman microscopy uses laser light to elicit the chemical and microstructural characterization of materials (Campion 2018).

Although the presence of talc particles found in ovarian cancer tissue does not prove that the talc played a causal role, when considered with the other lines of evidence supporting causation discussed in this report, the presence of talc in ovarian cancer tissue is certainly consistent with causation and provides additional evidence in support of a causal relationship between talcum powder products and ovarian cancer.

X. EPIDEMIOLOGICAL DATA REGARDING TALC USE AND OVARIAN CANCER:

As detailed below, there is consistent evidence from multiple observational studies, pooled analyses, and meta-analyses that exposure to talcum powder products is associated with an increased risk of ovarian cancer. When combined and considered with the biological evidence described above, this consistent epidemiologic data from multiple studies provides strong evidence that the association is, in fact causal.

Although occasional studies have not found talc powder applied to the perineum or contraceptive diaphragms[4] to be a significant risk for developing ovarian cancer, as detailed below, most have found an association, and the cumulative evidence from these studies, when considered with the other lines of evidence discussed above, provides strong and compelling evidence of a causal association.

XI. CASE-CONTROL STUDIES

Henderson first observed talc particles embedded in both ovarian tumors and normal ovaries (Henderson 1971). The first epidemiologic study on genital talc use and the risk of ovarian cancer was a case-control study by Cramer et al. (Cramer 1982). In this study, 215 women with epithelial ovarian cancer and 215 age-matched controls were questioned about talc use on the perineum and/or on sanitary napkins; 42.8% of ovarian cancer patients reported regular use of talc (prior to developing ovarian cancer) compared to 28.4% of controls, with an odds ratio (OR) of 1.92 (95% confidence level (CI) 1.27-2.89). The greatest risk in this study occurred in women who had used talc powder both directly on their perineum and on sanitary napkins compared to women who had no history of talc powder use; the odds ratio was 3.28 (CI 1.68-6.42). Of note, Cramer et al. did not exclude women from the control group who had a history of hysterectomy or other "pelvic surgeries" if the patient had intact ovaries by self-report. This could potentially lead to an underestimate of the risk of talc and ovarian cancer, as the controls may have had other confounding factors. They did control for confounding factors such as age, parity, religion, education, age of menarche, oral contraceptive use, hormone replacement therapy and smoking history.

While case control studies may have limitations with selection bias, Cramer et al. state "Our sample of cases represents more than 50% of ovarian cancer cases diagnosed

[4] It is likely that studies based on talc with diaphragm use are generally limited to use by women for birth control purposes. This will not capture use before or after the women's use of diaphragms for contraceptive purposes, a potential of multiple years that will not be captured in the study. Even for the years when women are using diaphragms, it is likely they are not using diaphragms for birth control on a daily basis. Therefore, diaphragm studies are likely to be biased toward the null; i.e., likely to understate talc exposure, and for that reason are likely to fail to detect an association that actually exists or understate the magnitude of risk.

in Boston residents in the study period. Therefore, it is difficult to conceive of a plausible bias in the selection of cases that would yield this excess use of talc." (Cramer 1982)

In additional to the Cramer 1982 study, numerous other case-control studies addressing talc use and ovarian cancer have shown statistically significant odds ratios greater than 1, indicating talc use is associated with an increased ovarian cancer risk (Harlow 1989, Booth 1989, Harlow 1992, Chang 1997, Cook 1997, Green 1997, Godard 1998, Cramer 1999, Gertig 2000, Ness 2000, Mills 2004, Merritt 2008, Wu 2009, Moorman 2009, Rosenblatt 2011, Kurta 2012, Houghton 2014, Wu 2015, Schildkraut 2016, Cramer 2016).

In a 1983 letter to the editor in JAMA in response to the 1982 Cramer study, Hartge and Hoover state that they found an association between genital talc use and ovarian cancer with a RR of 2.5, but the sample size was small (7 cases to 3 controls), resulting in a wide confidence interval (0.7-10.0). They did not find an association between ovarian cancer and body talc use or talc use on diaphragms, but again the sample sizes were small (Hartge 1983). Similarly, a study published by Tzonou et al. in 1983 showed no association between perineal talc use and ovarian cancer (RR 1.05; CI 0.28 to 3.98) but the frequency of reporting talc use was low in the study population, thus the wide CI (Tzonou 1983).

Whittemore et al. published a case-control study in 1988 that showed a RR of perineal talc use and ovarian cancer of 1.40, with a p value of 0.06. They did not see an increased risk of ovarian cancer in women who used talc on sanitary napkins or diaphragms. They did see an increased risk of ovarian cancer in women who used perineal talc for 1 to 9 years compared to those who used it for a shorter period (RR 1.60, p=0.05, CI 1.00-2.7) but did not see an increase with perineal talc users greater than 10 years (RR 1.11, p=0.61, CI 0.74-1.65). A strength of this study is that participants were not only asked about their history of talc use, but also about their history of cigarette smoking, coffee and alcohol consumption, thus addressing recall bias. A possible limitation of this study is the fact that the control group was a combined group of two separate control groups: one hospital based from the hospitals where the cases were admitted, and one community based. It was not described for what conditions the hospital controls were admitted (Whittemore 1988).

In 1989 Booth et al. published a study that showed an increased risk of ovarian cancer in daily talc users (RR 1.3, CI 0.8-1.9) and weekly talc users (RR 2.0, CI 1.3-3.4) as opposed to monthly (RR 0.7, CI 0.3-1.8) and rare (RR 0.9, CI 0.3-2.4) users. There were limitations of this study, however; participants were limited to women younger than 65 who had been diagnosed within the two years prior to interview. The data was adjusted for age in 5 year stratas and socio-economic status, but socio-economic status was based upon husband's career if married and participant's career if never married. Strengths, however, included queries of hormone replacement therapy, type of contraceptive use, and duration of oral contraceptive use; this helps to address recall bias. Additionally, hospital-based controls admitted for gynecologic disease and breast cancer,

among other diseases, were excluded and hospital admission diagnoses were listed (Booth 1989).

Harlow's 1992 study included 235 women with epithelial ovarian cancer and compared them to 239 control women matched for age, race and residence. After adjusting for age, parity, weight, education, marital status, religion, use of sanitary napkins and douching, it was found that talc use increased the ovarian cancer risk by 50% (OR=1.5, CI 1.0-2.1). Harlow's 1992 study also involved a dose-response effect; duration and frequency of perineal talc use was calculated into lifetime talc applications. Lifetime application ORs, when compared to control women with no perineal talc exposure, were 1.3 for <1000 (CI 0.7-2.7), 1.5 for 1000-10,000 (CI 0.9-2.4) and 1.8 for >10,000 (CI 1.0-3.0) (Harlow 1992). A dose response effect is a consideration in assessing causation. Harlow, Terry (2013) and Wu (2015) studies provide clear evidence of a dose effect. Particular strengths of the Harlow study are the number of potential confounding factors adjusted for and the detailed history on type of use and duration of use. Women with body exposure (non-genital) were considered non-exposed. Additionally, in the Harlow study, women were also asked about dietary and smoking histories, which helps to address potential recall bias.

Rosenblatt et al. published a study in 1992 that showed an increased risk of ovarian cancer with talc use (OR 1.7, but a small sample size with CI 0.7-3.9) (Rosenblatt 1992). In the Rosenblatt study, participants were also asked about oral contraceptive use and hormone replacement therapy, which helps to address potential recall bias. Another study published in 1992 by Chen et al. evaluated the association between talc use and ovarian cancer in a Beijing population. They found a RR of 3.9 in women with a history of use greater than 3 months, but the sample size was small with a 95% CI of 0.9-10.63. They also included dusting powder to the lower abdomen as well as perineum (Chen 1992), which would likely understate the magnitude of the association.

A 1997 study published in the journal *Cancer* by Chang et al. analyzed 450 patients with either ovarian borderline tumors or invasive ovarian carcinomas and showed an increased risk of tumor in women with either direct perineal application of talc or talc use on sanitary napkins (OR=1.42 after adjusting for age, parity, tubal ligation, hysterectomy, duration of oral contraceptive use, length of breastfeeding after pregnancy, and family history of ovarian cancer CI 1.08-1.86). For invasive ovarian carcinomas, the adjusted OR was 1.51 (CI 1.13-2.01). For borderline tumors, the adjusted OR was 1.24 (CI 0.76-2.02) (Chang 1997). The authors found that a borderline-significant association between duration of talc exposure and risk (OR 1.09, 95% CI 0.98-1.21, per 10 years of exposure). No significant association was found between frequency of exposure and risk. In comparing invasive and borderline carcinomas, risk remained elevated for both carcinoma types. The study did not assess for non-genital talc use. A particular strength of this study is that the same questions regarding talc use were asked about cornstarch use; they found no significant risk of ovarian cancer with cornstarch use (OR 0.31, CI 0.06-1.66), although only 1% of the populations reported using cornstarch (Chang 1997). Still, this helps to reconcile potential confounding risk factors of ovarian cancer in people more likely to use perineal powder. The interviews with participants also included taking

histories on oral contraceptive use and hormone replacement therapy, which helps to address recall bias.

Cook et al. also published a study in 1997 that evaluated 313 women with epithelial ovarian tumors (both invasive and borderline) and 422 controls. Only white women were included. They found that there was an increased risk of ovarian cancer with direct perineal powder dusting of 60% (OR=1.6, CI 1.1-2.3) and 90% (OR=1.9, CI 1.1-3.1) for genital deodorant sprays sprayed directly onto the perineum. Lifetime number of talc applications provided evidence of dose-response: a statistically significant increased risk (OR=1.7, CI 1.0-2.9 for less than or equal to 500 applications, OR=2.6, CI 0.9-7.6 for greater than 500 applications). A strength of this study is that participants were asked about smoking and contraceptive use, which helps to address recall bias. A limitation of this data is that all types of powder were included, such as cornstarch, "baby powder," "deodorant powder," and "scented body/bath powder." However, the authors state, "No specific type of powder used for perineal dusting, diaphragm storage, or on sanitary napkins was strongly related to ovarian cancer risk, although there was a suggestion of an elevated risk associated with any use of talcum powder and bath/body powders (RR = 1.6, 95 percent Cl 0.9-2.8, and RR = 1.5, 95 percent Cl 0.9-2.4, respectively)." (Cook 1997)

In 1997, an Australian study performed by The Survey of Women's Health Study Group enrolled 824 women with epithelial ovarian tumors, both invasive and borderline, and 855 controls. They found that the risk of ovarian cancer was highest among women who were talc users and had not undergone surgical sterilization (RR=1.3, CI 1.1-1.7) after adjusting for age, parity, duration of oral contraceptive use, BMI, smoking, education and family history of ovarian cancer. The risk was lowest in women who had not applied talc to their perineum and had either a tubal ligation or hysterectomy (RR=0.6, CI 0.50-0.84) (Green 1997). Because tubal ligation limits transport of talc fibers to the ovary, this study, with a finding of a protective effect in women with tubal ligation, provides an important piece of additional evidence. Strengths of this study include high response rate (90% of cases and 73% of eligible controls) and the verification of past surgical procedures by contacting participants' surgeons. Additionally, participants were asked questions about other potential exposures such as smoking histories and pelvic inflammatory disease, which helps to address recall bias. Limitations include a lack of data on quantity of talc use.

In 1999, Wong et al. published a paper that did not show a consistent association with talc powder and ovarian cancer, evaluated by length of use as follows: talc use for 1-9 years (OR 0.9; 95% CI 0.6, 1.5), 10-19 years (OR 1.4; 95% CI 0.9, 2.2), or more than 20 years (OR 0.9; 95% CI 0.6, 1.2). This was after adjustment for age at diagnosis, parity, oral contraceptive use, smoking history, family history of epithelial ovarian cancer, age at menarche, menopausal status, income, education, geographic location, history of tubal ligation, and previous hysterectomy. However, this study would tend to understate the magnitude of an association with genital talc use because it included talc use on thighs as well as genitals. The study used hospital controls, which raises a question of whether the controls were comparable to the cases (Wong 1999).

As part of Cramer et al.'s 1999 study, 563 women with newly diagnosed epithelial ovarian cancer were compared to 523 controls, and showed that perineal talc users had a significantly increased odds ratio for epithelial ovarian cancer (OR=1.60, CI 1.18-2.15). The effect of talc use was even stronger for invasive serous carcinoma (OR=1.70, CI 1.22-2.39). This was after adjusting for age, parity, oral contraceptive use, body mass index and family history of breast or ovarian cancer. The higher risk for women with invasive serous carcinoma was replicated in other studies, and this is an important finding in these studies because of its specificity. In addressing potential recall bias, Cramer et al. state, "…recall bias seems more likely to affect exposures that have occurred over a short term than those that have occurred over a long term. Since average duration of talc use exceeded 20 years in both cases and controls in our current study, genital talc exposure may be less likely to be subject to recall bias… It also seems reasonable that selective recall would lead to cases reporting all types of talc exposure more frequently than controls, but our study found that cases did not report a significant excess of talc use in non-genital areas compared to controls. Finally, if recall accounted for the association, one would expect little variation in the odds ratios by histologic type of ovarian cancer…. Regarding potential bias from confounding, we found no evidence that genital talc exposure varied by key risk factors for ovarian cancer such as age, parity or [oral contraceptive] use and little variability of the association by these and other variables." (Cramer 1999)

Ness et al.'s 2000 study evaluated 767 women with ovarian epithelial borderline tumors and ovarian invasive cancer compared to 1367 controls. Consistent talc use, defined as at least once per month for six or more months, increased the ovarian cancer risk by 50% (OR=1.5, CI 1.1-2.0) when applied to the perineal area directly and increased the risk by 60% (OR=1.6, CI 1.1-2.3) when used on sanitary napkins. This is after adjusting for age, parity, tubal ligation, hysterectomy, duration of oral contraceptive use, breast feeding and family history of ovarian cancer (Ness 2000). One explanation of the increased risk of talc use on sanitary napkins is that sanitary napkins may keep a larger amount of talc closer to the vagina over the course of several hours, thus increasing the risk of entry to perineum, while talc directly applied to the perineum may more easily disperse, however, many studies have failed to show an increased risk in ovarian cancer in participants whose only exposure to talc was on sanitary napkins. The strengths of this study include addressing multiple confounding factors. No dose-response was found; weaknesses include that only duration information was available, and genital/rectal talc use durations reported were combined with duration of use on the feet. Additionally, women who used just once per month were categorized as a user. These weaknesses may cause an underestimation of risk, and may have accounted for the lack of dose-response found.

Mills et al. published a study in 2004 that evaluated the association between talc use and ovarian cancer among 256 cases of ovarian cancer as compared to 1122 controls. Women diagnosed with invasive epithelial ovarian cancer with a history of genital talc use had an increased risk of 51% (OR=1.51, CI 1.07-2.12). This increased risk increased to 77% (OR=1.77, CI 1.12-2.81) for women diagnosed with invasive serous carcinoma.

Dose-response effects were also found. Increasing frequency of use was associated with increasing risk; women who reported use 4–7 times per week had a 74% elevation in epithelial ovarian cancer risk (p for trend = 0.015). However, the risk decreased between the second and third categories of use (from "rarely to several times per month" and "1-3 times per week" at 1.34 (CI 0.87-2.08) to 1.16 (CI 0.74-1.81), respectively). Duration of use of talc was also associated with increased risk, although the risk peaked among those reporting 4–12 years of use and declined somewhat among those reporting longer duration of use (p for trend = 0.045). Cumulative use also demonstrated an uneven association with risk of epithelial ovarian cancer in that the point estimates peaked in the second and third quartiles of intensity but declined in the highest quartile of use. These findings were after adjusting for age, race/ethnicity, duration of oral contraceptive use and duration of breast feeding. Yet, there wasn't adjustment for first relative history of breast or ovarian cancer, pregnancy history, parity, BMI, hysterectomy, tubal ligation or hormone replacement therapy; according to the authors, the Hosmer-Lemshow goodness-of-fit tests revealed that after terms for duration of oral contraceptive use and duration of breast-feeding were added to the models, fit was not improved by the addition of these variables, nor were the estimated odds ratios altered by the addition of several of these variables (Mills 2004). However, the fact that participants were queried about other possible exposures such as hormone replacement therapy helps to address potential recall bias.

In Wu et al.'s 2009 study, women were found to be at increased risk of ovarian cancer if they had a history of prior perineal talc use, with the risk increasing significantly in those with long term (20+ years) and frequent (at least daily) use with a relative risk of 2.08 (CI 1.34-3.23), i.e., a dose effect. The authors did find an increased risk in women who used talc on sanitary napkins (RR 1.61, CI 0.93-2.78), underwear (RR 1.71, CI 0.99-2.97) and diaphragms/cervical caps (RR 1.14, CI 0.46-2.87). There was a stronger association between talc use and serous ovarian cancer; the relative risk with any talc use was 1.70 (CI 1.27-2.28). Strengths of this study include the adjustment for multiple possible confounding factors (age, race/ethnicity, education, age of menarche, parity, oral contraceptive use, family history of ovarian or breast cancer, menopausal status and tubal ligation). Another strength was that participants were queried about NSAID and endometriosis histories, helping to address potential recall bias. The authors mention in their discussion that the participation response was "modest," possibly leading to selection bias (Wu 2009).

Rosenblatt et al. published a study in 2011 that showed an overall increased risk of ovarian cancer in women who used talc after bathing (OR=1.27, CI 0.97-1.66) with a more pronounced risk in women diagnosed with mucinous borderline tumors (OR=1.78, CI 0.98–3.23) and serous borderline tumors (OR=1.47, CI 0.85-2.55) (serous borderline tumor illustrated in Figure 3). They did not see an increased risk by extent of use, defined as years in which powder was used, or as lifetime number of applications. There was no alteration in the risk of ovarian cancer associated with other types of powder exposure such as sanitary napkins or diaphragms. This study did not find an increased risk of invasive serous carcinoma (OR 1.01, CI 0.69-1.47). (Rosenblatt 2011) A strength of this

study is that participants were queried about other potential exposures (smoking, alcohol and endometriosis histories), which helps to address recall bias.

In 2012, Kurta et al. evaluated talc use and the risk of ovarian cancer, although their main focus of the study was the associated risk of ovarian cancer with fertility drug use. They found a OR of 1.40 (CI 1.16-1.69). Since talc was not the primary focus of this study, duration of use was not considered; participants were categorized as talc users if they had ever used talc versus never-users. Perineal talc use was only generally defined as dusting powder or deodorizing spray on the genital or rectal areas, sanitary napkins, underwear, or diaphragms or cervical caps (Kurta 2012). A strength of this study is that its main focus was on fertility drug use; participants were asked about exposures such as fertility treatments and hormone replacement therapy, which helps to address potential recall bias.

Wu et al. published a paper in 2015 that evaluated talc use and invasive ovarian cancer in white, Hispanic and African American women. They found that talc use was more common in African-American women (44.1%) than in non-Hispanic whites (30.4%) or Hispanics (28.9%) (p=0.001). The results showed ORs of 1.41 for white women (CI 1.21-1.67), 1.77 for Hispanic women (CI 1.20-2.62) and 1.56 for African American women, although the CI for African American women was 0.80-3.04. Overall, the OR was 1.46 (CI 1.27-1.69). However, the response rate and sample size for this study was somewhat small, and participants with less than one year of use were categorized as never users (Wu 2015).

In 2016, Schildkraut et al. published a paper as part of the African American Cancer Epidemiology Study (AACES), a case-control study of epithelial ovarian cancer in African American women. According to the authors, due to the relatively small number of women who reported having only used genital powder (43 cases and 44 controls), the authors merged this exposure category with those who reported use of both non-genital and genital powder, creating an exposure category of “any” genital powder use, but separately evaluated the categories as “only” or “any” genital powder use. They reported an increased risk of ovarian cancer in “any” genital powder users (OR=1.44, CI 1.11-1.86) and noted a statistically significant dose response effect for both duration of use and lifetime applications. A strength of this study was adjustment for multiple confounding factors such as age, education, BMI, parity, tubal ligation, OCP use, first degree relative with breast or ovarian cancer, and interview year (taking into account litigation cases in the year 2014). Participants were also asked about hormone replacement therapy, another potential exposure, thus helping to address potential recall bias. A weakness of this study is that participants were considered “regular users” if they reported using cornstarch, baby or deodorizing powders at least one time per month for at least 6 months, and “never users” if they did not, leading to possible misclassification that would bias toward the null (Schildkraut 2016).

The totality of the results of the case-control studies support a causal link between talc and ovarian cancer. When observational studies find an increased risk of disease with a certain exposure, the possible reasons are chance, bias, confounding and causation.

There is a general consistency of these individual studies; the ORs have been of similar magnitude in studies spanning different decades, in different populations, with different study designs, by different investigators, over different continents and with adjustment for multiple confounders. Therefore, the possibility that the association between perineal talc use and ovarian cancer is due to chance is extremely unlikely.

Although retrospective case-control studies potentially have an element of recall bias and other potential biases, again, the consistency of results across these studies and populations makes recall and other bias an unlikely explanation. During the period that the majority of studies were conducted, public awareness of the link between talc and ovarian cancer was limited. There is also a much stronger and statistically significant association of perineal talc use and ovarian cancer in studies that compared all-body talc use to perineal use. The finding in some studies that serous carcinoma has a stronger association with perineal talc exposure than other histologic subtypes of ovarian cancer also argues against recall bias, as participants are very unlikely to have knowledge about the histologic subtyping of ovarian cancer. In addition, in studies where participants are asked to recall multiple exposures, not just talc exposure, this will minimize the risk of recall bias because it is unlikely that participants will differentially recall talc exposure but not other exposures, especially if they are blinded to the study hypothesis. Studies using trained interviewers, structured interview questionnaires, and blinding of both study participants and the interviewers to the study hypotheses will also limit the potential for recall bias.

Selection bias (which can arise based on differential participation rates or other differences between comparison groups) accounting for the results across studies is also unlikely. To see such consistent associations between perineal talc use and ovarian cancer, there would need to be strong associations between participation and perineal talc use, and strong differences amongst cases and controls due to selection bias only - this would be extremely unlikely to produce such large biases across studies. Most studies adjusted for confounders, with the majority adjusting for age, BMI, and parity among others. With chance, bias, and confounding being unlikely explanations for the association of perineal talc use and ovarian cancer across multiple studies, this leaves causation as the most likely explanation.

XII. COHORT STUDIES

The talc literature includes several cohort studies reporting the relative risk for perineal talc use and risk of ovarian cancer, including the Nurses' Health Study, the Women's Health Initiative and the Sister Study (Gertig 2000, Gates 2008, Gates 2010 and Gonzalez 2016). There were several important limitations of these studies to adequately capture risk of ovarian cancer based on the methodology used by the researchers to assess talc exposure.

The Gertig study evaluated prospective cohort data from 78,630 women, and although there was a 12% overall increased risk of ovarian cancer in women with a history of daily genital talc use, this was not statistically significant. Yet, the investigators

reported a statistically significant increased risk of invasive serous carcinoma (RR=1.4, CI 1.02-1.91) after adjusting for age, parity, duration of oral contraceptive use, post-menopausal hormone use, tubal ligation, BMI and smoking (Gertig 2000). Additionally, the lack of statistical significance of overall ovarian cancer risk may be due to several important limitations with this study, including the fact that the question of talc use was only in one questionnaire in 1982 and did not include questions on duration of use. Thus, a person who used talc just a few times would be included with women who used talc daily over a long duration, and this will have the effect of understating the risk. In fact, in a follow-up 2008 report, Gates et al. noted that since talc exposure was only referred to once in questionnaires, it is possible that some participants were misclassified with respect to their talc use or that some women may have started talc use after 1982 and thus these women would not be included in the talc user group (Gates 2008). This would understate the risk and decrease the calculated statistical significance of talc-related ovarian cancer. An additional review of the Nurses' Health Study published by Gates et al. in 2010 studied 876 cases of ovarian cancer and talc use, although this was not the primary focus of the study. This study found an overall increased risk of ovarian cancer with talc use (RR=1.06), but found an increased risk for mucinous tumors (RR=1.50) (Gates 2010) (mucinous carcinoma illustrated I Figure 6). Again, the weaknesses in the study include the fact that talc use was only queried once in 1982, and the authors state themselves that the limited data on talc use may have influenced the observed association with ovarian cancer.

Cohort studies like the Nurses' Health Study, Women's Health Initiative Study and the Sister Study have some drawbacks when studying rarer diseases compared to case-control studies that have been described above. Cohort and case-control studies are both observational, and both have strengths and limitations. Cohort studies begin when all participants are free of the disease in question. After a follow-up period, those that have the disease being studied are compared by exposure risk being studied to those who did not develop the disease. Although this helps to ensure exposure predates disease, there may be a lack of data if the disease is rare or if there is a long latency period between exposure and disease presentation/diagnosis, as is the case of ovarian cancer and talc. In contrast, in case-control studies, patients already have the disease being studied and are compared to controls who do not have the disease with a focus on the rates of exposure to the agent of interest (here, talcum powder products) in the cases as compared to the controls. A possible limitation of case-control studies in the context of ovarian cancer and talc is the fact that exposure to talc is self-reported and subject to potential recall bias.

The case-control studies may unavoidably have recall bias, as talc use was self-reported by participants. In their 2018 meta-analysis discussed below, Penninkilampi et al. noted that in some studies, interviewers were not blinded to cases and controls and many studies did not describe whether their controls had a personal history of previous ovarian cancer. However, they also noted that in general, controls were well matched to cases by other possible confounding factors such as age, geographic, location and ethnicity (Penninkilampi 2018).

In the 2008 Gates paper, women with certain variants in glutathionine S-transferase M1 (GSTM1) and/or glutathionine S-transferase T1 (GSTT1) were shown to have a higher risk of talc-associated ovarian cancer. Glutathione S-transferases catalyze the conjugation of glutathione to numerous potentially genotoxic compounds. Individuals with homozygous deletions of GSTM or GSTT have reduced or no glutathione S-transferase activity and may be unable to eliminate electrophilic carcinogens as efficiently (Coughlin 2002). The 2008 Gates study included 1,175 cases and 1,202 controls from a case-control study and 210 cases and 600 controls from the prospective Nurses' Health Study. Participants were genotyped for the GSTM1 and GSTT1 gene deletions and three NAT2 polymorphisms. Regular talc use was associated with increased ovarian cancer risk in the combined study population (relative risk=1.36, CI 1.14-1.63; p-trend<0.001). In the pooled analysis, the association of talc and ovarian cancer was stronger among women with the GSTT1-null genotype (p-interaction=0.03), particularly in combination with the GSTM1-present genotype (p-interaction=0.03). There was no clear evidence of an interaction with GSTM1 alone or NAT2. Without talc exposure, these genes were not clearly associated with risk of ovarian cancer (Gates 2008). The specificity of the findings linking the genetic polymorphisms with ovarian cancer subtype most associated implicates yet another aspect of the Bradford Hill viewpoints.

As previously detailed, the Nurses' Health Study also showed that genital talc use was associated with lower levels of anti-MUC1 antibodies, which has been associated with an increased risk of ovarian cancer. As part of the Nurse's Health Study, Pinheiro et al. published a paper in 2010 that showed increasing anti-MUC1 antibody levels were associated with a nonsignificant trend for a lower risk of ovarian cancer with highly significant heterogeneity by age (p-heterogeneity=0.005). The authors concluded that anti-MUC1 antibodies evaluated several years prior to diagnosis may be associated with lower risk of subsequent ovarian cancer in women less than 64 years old at assessment (Pinheiro 2010). Cramer et al. 2005 study showed factors which increase the levels of anti-MUC1 antibodies lower the risk of ovarian carcinoma (Cramer 2005). These findings provide evidence that a plausible mechanism for talc-associated ovarian cancer is a down-regulated immune response to MUC1, and thus an immune tolerance of an emerging MUC1-expressing tumor.

The Women's Health Initiative Observational Study (WHI-OS) did not report a statistically significant increased risk of ovarian cancer with talc use (Houghton 2014). In that study, 61,576 women were enrolled and 429 developed ovarian cancer during follow-up. The study did find a 12% increased risk of ovarian cancer in perineal talc users (RR=1.12, CI 0.92-1.36), but it was not statistically significant. However, the risk of developing serous carcinoma was increased by 18% (RR=1.18, CI 0.89-1.56), and by 13% for invasive serous carcinoma (RR=1.13, CI 0.84-1.51). Additionally, 101 cases were categorized histologically as "other," including tumors that were self-reported, not validated and potentially may not have even been primary ovarian tumors. This would bias the risk estimate of talc use in ovarian cancer in this study toward the null by including cancers or other tumors potentially from other sites; in other words, non-specific cancer types may have been included that are not known to have an association with talc use. Another weakness of the study is that although the authors did evaluate the

effect of duration of use of genital talc on the risk of ovarian cancer, they did not evaluate frequency of use. Thus a woman who used talc for twenty years once a month would be treated the same as a woman who used it every day for twenty years. This will tend to understate or obscure the true risk of long term, frequent use. The study also was of an older age group (50-79) who were post-menopausal at time of enrollment, which adds selection bias.

Another study in which the effect of talc use on the risk of ovarian cancer is likely diluted or understated is the Sister Study, published by Gonzalez et al. in 2016. In this study, there was no reported association between perineal talc use and subsequent ovarian cancer. The study only enrolled women with a full or half-sister who had been diagnosed with breast cancer. BRCA1 and BRCA2 mutations are associated with a markedly increased risk of both breast and ovarian cancer, and in the Sister Study, women were not tested for this mutation. Most of the ovarian cancers associated with BRCA mutations are of the invasive serous subtype, the same subtype most strongly associated with talc use in prior studies. By not testing the women for the genetic mutation, the Sister Study analyzed a population of women with an increased risk of having a BRCA mutation (by having a first degree relative, or sister/half-sister, with breast cancer), a significant confounding factor that was not considered. Another limitation of this study is that the mean follow-up was 6.6 years, a very short period considering the generally long latency period of ovarian cancer. The Sister Study did find an increased risk in ovarian cancer in women who douched, providing evidence supporting the link between particulate route of access to the ovary/fallopian tube. The histologic subtype of the ovarian cancer was also not evaluated. Further, similar to the other cohort studies, the Gonzalez 2016 study failed to adequately capture both duration and frequency of talc exposure as participants were only asked if they used talc in the last 12 months.

XIII. META-ANALYSES REGARDING TALC USE AND OVARIAN CANCER:

Meta-analyses are an important tool that combines study results from multiple studies to develop a single result that has greater power to detect a more precise estimate of risk. Several meta-analyses have been published on the association between talc use and ovarian cancer, all showing an increased risk (Harlow and Cramer 1992, Gross and Berg 1995, Cramer and Harlow 1999, Huncharek 2003, Langseth 2008, Berge 2018, Penninkilampi 2018).

In 1992 Harlow and Cramer published combined results from six case-control studies of the association between talc use and ovarian cancer that were performed between 1982 and 1989. The association was statistically significant (OR=1.3, CI 1.1-1.6) (Harlow 1992). In 1995, Gross and Berg published a meta-analysis that included the six case-control studies evaluated in the 1992 Harlow and Cramer paper, plus three additional studies. This produced a statistically significant increased risk (OR=1.27, CI 1.09-1.48) (Gross 1995). Of note, this study was supported in part by Johnson and Johnson, raising the issue of funding bias.

Cramer published another meta-analysis in 1999 that included the nine studies in Gross and Berg's 1995 paper plus five additional ones performed through 1999. The overall risk of ovarian cancer in talc users was found to be increased at 36% (OR=1.36, CI 1.24-1.49) (Cramer 1999).

Huncharek et al. performed a meta-analysis in 2003 that added five new studies and included all of the previous studies except the 1983 Hartge and 1996 Shushan studies. The OR in this study was 1.33 (CI 1.16-1.45). Interestingly, the authors concluded that even with this statistically significant OR, the data "do not support the existence of a causal relationship" between talc use and ovarian cancer (Huncharek 2003). In a subsequent paper published by Huncharek et al., support from Johnson and Johnson and Luzanec America was acknowledged (Huncharek 2007), raising the issue of funding bias.

Langseth et al. published a comprehensive meta-analysis in 2008 of the risk of ovarian cancer associated with talc use. The combined OR was 1.35 (CI 1.26-1.46), and specifically 1.4 for population-based studies (CI 1.29-1.52), the less potentially biased type of study. Langseth et al. also noted that the risk of serous ovarian tumors in particular with talc use may be greater (Langseth 2008).

In 2016, Cramer published a retrospective case-control study that incorporated data from three enrollment phases (1992-1997, 1998-2002 and 2003-2008) and combined data from the Nurses' Health Study (Gates 2008) and data from participants in the Ovarian Cancer Association Consortium (OCAC, Terry 2013). The study found a statistically significant increased risk of invasive serous, invasive endometrioid and serous borderline ovarian tumors in women who were genital talc users, with the highest risk (OR=2.33 (CI 1.32-4.12) and OR=2.57 (CI 1.51-4.36) for pre- and postmenopausal women, respectively) with the greatest lifetime exposure, as defined by "talc-years," or number of applications per year multiplied by years of use. A dose-response was most prevalent for invasive serous carcinoma. This study is important as evidence supporting an association between talc and ovarian cancer as the authors analyzed case-control data collected over 16 years in 2,041 epithelial ovarian cancer cases and 2,100 age- and-residence-matched controls. As the authors state, they "addressed issues related to definition of the exposure, bias and confounding, effect modification, histologic heterogeneity, and dose-response. Talc used regularly in the genital area was associated with a 33% increase in ovarian cancer risk overall." (Cramer 2016)

Berge et al. published another meta-analysis in 2018 that found a summary RR of 1.22 (CI 1.13-1.30). They found that the association between talc and ovarian cancer was stronger in case-control studies (RR 1.26, CI 1.17-1.35) than cohort studies (RR 1.02, CI 0.85-1.20). The limitations of the cohort studies are discussed above; limitations of case-control studies are recall bias and selection bias. Addressing the latter, Berge et al. found a higher summary risk estimate in hospital-based case-control studies compared to community-based case-control studies, but this difference was not statistically significant. Recall bias can be present in case-control studies, however, Berge et al. found the greatest association between genital talc use and serous carcinoma (RR 1.24, CI 1.15-

1.34). This would argue against recall bias, as participants would likely not know the categorization of epithelial ovarian tumors, nor the fact that invasive serous carcinoma has been shown to have the strongest association in the majority of studies.

Penninkilampi et al. published a meta-analysis in 2018 that found any perineal talc use was associated with an increased risk of ovarian cancer (OR 1.31, CI 1.24-1.39). They found a dose-response effect with greater than 3600 lifetime applications (OR 1.42, CI 1.25-1.61) compared to less than 3600 lifetime applications (OR 1.32, CI 1.15-1.50). Similar to the Berge 2018 study, an association was found in the case-control studies (OR 1.35, CI 1.27-1.43) but not in the cohort studies (OR 1.06, CI 0.90-1.25). However, Penninkilampi et al. did find an association in cohort studies between talc use and invasive serous carcinoma (OR 1.25, CI 1.01-1.55). (Penninkilampi 2018)

XIV. POOLED STUDY REGARDING TALC USE AND OVARIAN CANCER:

The meta-analyses discussed above summarize previously published data and thus have increased statistical power for a more precise estimate of effect on talc in ovarian cancer risk (Cohn 2003). However, the strength of meta-analyses depends on the quality of the previously published data analysis. In comparison, a pooled study analyzes primary data from different studies/researchers. The Terry 2013 study is a retrospective pooled study from eight population-based case-control studies from OCAC. One advantage of pooled studies is the ability to include a large sample size; Terry et al. included 8,525 cases of ovarian, fallopian tube or perineal cancer and 9,859 controls. Some of the included OCAC studies had previously reported on powder use (Chang 1997, Cramer 1999, Merritt 2008, Moorman 2009, and Rosenblatt 2011), and according to Terry et al., three of these provided data for the pooled 2013 analysis that had not been included in the previous publications. The other three studies had not previously published their genital powder data (Goodman 2008, Lo-Ciganic 2012, Pike 2004). The pooled analysis showed an OR for genital talc use and epithelial ovarian cancer of 1.24 (95% CI 1.15-1.33) after adjustment for age, oral contraceptive use, tubal ligation, BMI and race/ethnicity (Terry 2013). This is consistent with the majority of meta-analyses and individual studies.

A strength of a pooled study versus a meta-analysis is that pooled studies have increased standardization. As an example, the Terry 2013 study excluded participants that data was not available on regarding tubal ligation, oral contraceptive duration, parity or height and weight. This adjusts for study-specific differences in confounding factors. A weakness of pooled studies is that they are limited by the methods of original data collection; for example, Terry et al. state “Limitations of our pooled analysis include differences in the wording of questions about genital powder use between studies and the retrospective nature of the exposure ascertainment.” As Blettner (1999) stated, “Pooling decreases the variation caused by random error (increasing the sample size) but does not eliminate any bias (systemic errors).” In the 2013 Terry et al. study, classification between cases and controls differed between studies, as the women who were classified as genital powder users varied from “ever” use, “ever regular” use, to powder use for at least one year. However, Terry et al. conclude that if anything, this led to an underestimate of the true association for any given

study "[due to the fact that] exposure definitions are the same for cases and controls within each study, misclassification of genital powder exposure due to the question wording would be nondifferential…." (Terry 2013).

XV. ASBESTOS, TALCUM POWDER PRODUCTS, AND OVARIAN CANCER:

I have seen evidence that talcum powder products manufactured by Johnson & Johnson (J&J Baby Powder and Shower to Shower) contained and continue to contain asbestos, talc containing asbestiform fibers (e.g. talc occurring in a fibrous habit) heavy metals (such as cobalt, chromium, nickel) and fragrance chemicals (Longo et al. 2017 and 2018, Blount 1991, Blount Deposition 2018, Hopkins Deposition and Exhibit 2018, Pier Deposition and Exhibit 2018). Other than cobalt, which has been identified as a "possible" carcinogen, all of these constituents have been identified as known carcinogens by IARC (IARC 2012). It should be noted that National Institute for Occupational Safety and Health (NIOSH) has determined that "there is no safe level of asbestos exposure for any type of asbestos fiber" (NIOSH 1980). As part of my review and consideration of the evidence I have also reviewed Dr. Michael Crowley's opinion that "fragrance chemicals in Johnson & Johnson talcum powder products contribute to the inflammatory properties, toxicity, and potential carcinogenicity of the products." The presence of these constituents as part of the talcum powder product provides additional evidence of biological plausibility for talcum powder products to cause ovarian cancer.

Asbestos is a silicate mineral in polyfilamentous bundles. Other silicate minerals exist, such as talc, but asbestos is classified by its flexible fibers with small diameter and large length. The forms of asbestos are serpentine silicates ("sheet silicates") such as chrysotile, and amphibole silicates ("chain silicates") such as crocidolite, amosite, anthophyllite, actinolite, and tremolite (IARC Monograph). The carcinogenic properties of asbestos fibers depend on the length of the fiber (Stanton 1972) and its chemical composition, structure, and cell environment (Mossman 1998, Robledo 1999, IARC Monograph). Asbestos fiber surface reactivity with free radical generation has also been accepted as a mechanism of carcinogenesis (IARC Monograph). Asbestos-derived free radicals can lead to a variety of effects on cells including lipid peroxidation, DNA oxidation, TNF release, cell apoptosis, and increased uptake of asbestos fibers (Mossman 1983, Hobson 1990, Ghio 1998, Churg 1998, Gulumian 1999, Aust 1999, Upadhyay 2003, IARC Monograph). Asbestos fibers may directly cause the generation of ROS (IOM 2006) and indirectly cause ROS by inducing inflammation and macrophage activation (IARC Monograph).

It has long been generally accepted that asbestos exposure causes mesothelioma and lung cancer (Dement 1994, deKlerk 1996, Berry 2000). Approximately 125 million people around the world have been exposed to asbestos in work environments, and at least 90,000 people die each year from asbestos-related lung cancer, mesothelioma, or asbestosis (Burki 2009). The relationship between asbestos exposure and ovarian cancer had been less studied; however, in 2009, the IARC Monograph Working Group concluded that there is sufficient evidence to show that asbestos exposure can cause ovarian cancer (Straif 2009, IARC Monograph).

In the late 1960's, a suggested link between talc and ovarian cancer was made for the following reasons: first, talc powders were shown to contain asbestos (Cralley 1968); second, intraperineally placed asbestos in animals induced a proliferation of the ovarian mesothelial lining from one layer to multiple layers (Graham 1967). Of note, it was tremolite asbestos that was used by Graham, the same type of amphibole asbestos that is found in asbestos-contaminated talc. It is important to note that similar to talc being found on the ovarian surfaces of perineal talc users, asbestos fibers have been found in women whose household contacts worked with asbestos and in Norwegian paper and pulp workers (Heller 1996, Langseth 2007).

In 1972, Newhouse et al. published a study of the mortality of female asbestos workers and found at least 4 deaths due to ovarian cancer compared to an expected number of 0.6. During histological review of some of the pathology samples from these workers, there was evidence that another two deaths that had been registered as due to carcinomatosis were likely caused by ovarian cancer (Newhouse 1972).

Ten years later in 1982, Wignall et al. published a study that followed 535 women who were assembly workers that had direct crocidolite exposure during the manufacturing of military gas masks. The authors found 2 deaths due to ovarian cancer in women that were employed at the facility for less than 1 year, with a standardized mortality rate (SMR) of 1.77. Two ovarian cancer deaths occurred in women with a 1 year history of employment at the facility (SMR=2.11) and one ovarian cancer death in a woman with a 3 year history of employment (SMR=1.05). The authors noted that the expected number of deaths is low, making stable estimates of SMR difficult. However, the authors conclude that the "excess of deaths from carcinoma of the ovary was unexpected at the start of the study but appears to be related directly to exposure to asbestos" (Wignall 1982).

Also published in 1982 was a study by Acheson et al. that evaluated two groups of women exposed to asbestos who assembled gas masks in two separate facilities: 570 women at Blackburn (civilian respirators that contained chrysotile) and 757 women at Leyland (military respirators containing crocidolite). The study found a SMR in the crocidolite group for ovarian cancer of 2.75 (CI 1.42-4.81) and a SMR of 1.48 (CI 0.48-3.44) for the chrysotile group. The authors noted that the risk of ovarian cancer increased over time for up to 40 years post exposure (Acheson 1982).

A 1994 study by Rosler et al. examined mortality from ovarian cancer in a cohort of 616 women in Germany who had been occupationally exposed to asbestos. Although about 95% of asbestos used in Germany was chrysotile, the authors noted that they could not exclude a mixture containing crocidolite. Two deaths from ovarian cancer were observed, compared to an expected 1.8 (SMR 1.09, CI 0.13-3.95). (Rosler 1994).

In 1999, Germani et al. published a study of ovarian cancer mortality in 631 women workers in Italy who had been compensated for asbestosis. They found a total of nine ovarian cancer deaths (SMR 4.77, CI 2.18-9.04) which included four deaths in a subset of asbestos-textile workers (SMR 5.26, CI 1.43-13.47) and five deaths in the subset of asbestos cement workers (SMR 5.40, CI 1.75-12.61). (Germani 1999).

Also in 1999, Vasama-Neovonen et al. published a case-control study of ovarian cancer and occupational exposure in Finland. The Standardized Incidence Ratio (SIR) was 1.30 (CI 0.9-1.80) between ovarian cancer and "medium/high levels of asbestos," and the SIR was 1.1 (CI 0.8-1.3) for "low levels of asbestos." The SIR is obtained by dividing the observed number of cases of cancer by the expected number of cases in the general population. The type of asbestos fiber was not noted (Vasama-Neovonen 1999).

Again in 1999, Langseth et al. published a study of 4247 workers employed for at least one year between 1920 and 1993 in the Norwegian pulp and paper industry. 85% of them were paper or administration workers. The follow-up period for cancer was from 1953-1993. An excess risk of ovarian cancer was found (SIR = 1.50, CI 1.07-2.09). The SIR was highest among those younger than 55 years, and mostly among those working in paper departments. The type of asbestos fiber was not specified (Langseth 1999). Langseth et al. published a follow-up case-control study in 2004 that examined the association between asbestos exposure and ovarian cancer in this same cohort of female pulp and paper workers in Norway that had been found to have excess morbidity from ovarian cancer. In the case-control study, the odds ratio for occupational exposure to asbestos based on 46 cases of ovarian cancer was 2.02 (CI 0.72-5.66), although this was not statistically significant (Langseth 2004).

In 2000, Berry et al. published a study that evaluated the mortality of a cohort of over 5000 London asbestos factory workers, both men and women, who were followed for over 30 years since first asbestos exposure. The study classified exposure by degree (low, moderate and severe) and duration (2 years or less or more than 2 years). They assessed mortality by comparing the number of cohort deaths with the number of expected deaths in England and Wales based on sex, age and period. The study found that there was a significant increase of ovarian cancer in women with severe exposure for more than 2 years (SMR of 5.35) and an overall SMR for all exposure lengths of 2.53 (CI 1.16-4.8) (Berry 2000).

In 2005, Pira et al. published a cohort study of 1077 women with at least a one month history of employment between 1946 and 1984 at an asbestos-textile factory in Italy. A variety of asbestos types were used in this facility, including crocidolite. They followed up with the cohort in 1996. There were five deaths due to ovarian cancer with an overall SMR of 2.61 (CI 0.85-6.09), but there was a SMR of 5.73 for women with longer employment histories at the facility (greater than or equal to 10 years of employment). Among women with greater than or equal to 35 years since first employment exposure, the SMR was 5.37 (Pira 2005).

Also in 2005, Wilcsynska et al. published a study of 1470 Polish asbestos cement factory workers with a follow-up period from 1945 to 1999 and a SMR of ovarian cancer among workers of 3.76 (CI 1.38-8.18). The type of asbestos fiber was not specified (Wilcsynska 2005).

McDonald et al. published a study in 2006 that followed 567 people, mostly women, who had assembled gas masks in the Nottingham factory between 1940 and 1944 and showed

a SMR for ovarian cancer of 1.2 (CI 0.6-2.2). Gas masks assembled at this facility had filter pads that contained 20% crocidolite. As an aside, this study found that the first deaths due to mesothelioma happened a little more than 20 years after exposure, which is consistent with most other studies (McDonald 2006) and highlights the lengthy time interval between exposure and presentation of disease in asbestos-related mesothelioma.

In 2008 Reid et al. published a study of 2552 women and girls who lived in a Western Australia mining town between 1943 and 1992 where crocidolite asbestos was mined. They were not directly involved in mining but there was extensive environmental contamination of the town. They found a SMR for ovarian cancer of 1.52 (Reid 2008).

Reid et al. published a study in 2009 that followed the same cohort of 2552 women and girls in Western Australia with environmental exposure to crocidolite asbestos and added 416 women to the study that had worked in the Wittenoom crocidolite asbestos mines and mills. For the latter group, there wasn't an increased rate of ovarian cancer (SIR of 0.49, CI 0.01-2.74), but the authors noted that the "female Australian Blue Asbestos workers at Wittenoom mostly worked in the company offices, shop, and hotel. Their occupational exposure was unlikely to have been as high as that reported for women in the earlier cohorts, which may explain why no excess risk for ovarian cancer was observed" (Reid 2009).

Pukkala et al. published a study in 2009 on the incidence of ovarian cancer in women employed in various occupations in Denmark, Finland, Iceland, Norway and Sweden. One of the groups examined were plumbers, who are known to have occupational exposure to asbestos. Four ovarian cancers were found in this group of plumbers, with a Standardized Incidence Rate (SIR) of 3.33 (CI 0.91-8.52). Fiber type was not specified (Pukkala 2009).

Magnani et al. and Bertolotti et al. published studies in 2008 that followed the same cohort of former asbestos-cement workers who were employed at a facility in Casale Montferrato, Italy. A mix of crocidolite and chrysotile asbestos was used at this factory. They observed nine ovarian cancer deaths versus 4 expected (SMR of 2.27). In women who had 30 or more years of exposure, the SMR was 2.97 (Magnani 2008, Bertolotti 2008). Ferrante et al. published a study in 2007 that examined cancer mortality in the household contacts of men who worked at this facility; among women with exposure due to household contacts, there were 11 ovarian cancer deaths versus an expected 7.7, or SMR of 1.42 (CI 0.71-2.54). (Ferrante 2007).

I am aware of two meta-analyses, both published in 2011, that evaluated a link between asbestos and ovarian cancer. The first was published in 2011 by Reid et al. and analyzed fourteen cohort and two case-control studies of women with exposure to asbestos in their work environment. The majority of the cohort cases they evaluated are detailed above. The authors added a 2002 paper by Szeszenia-Dabrowska et al. that studied Polish women diagnosed with asbestosis and a 2004 paper by Mamo et al. that studied Turin asbestos textile factory workers (Szeszenia-Dabrowska 2002, Mamo 2004). The two case-control studies they evaluated were a 1992 study of Johns Hopkins patients by Rosenblatt et al. and a 2004 study

of Norwegian pulp and paper workers by Langseth et al., the same group of workers previously described above. Reid et al. concluded that although women "thought to have ovarian cancer" (not all cases of ovarian cancer were histologically reviewed and confirmed) had an increased rate if exposed to asbestos, the overall numbers were still small and further study was warranted as one misclassification could skew the data (Reid 2011).

The authors of the second 2011 meta-analysis, Camargo et al., included 18 studies. They did not include the 1992 Rosenblatt et al. study or the 2004 Langseth et al. study but added six others: a 1986 study of cement workers in the U.K. by Gardner et al., a 1989 study of friction material workers in the U.K. by Newhouse et al., a 2007 study of textile workers in the U.S. by Hein et al., a 2009 study of textile workers in the U.S. by Loomis et al., and two other 2009 studies by Harding et al. and Clin et al. The authors of this second meta-analysis came to a stronger conclusion that the findings were consistent with an association between asbestos exposure and an increased risk of ovarian cancer (Carmargo 2011).

Considering the consistency of these studies, the Bradford Hill viewpoints (strength of association, consistency, biological plausibility, etc.) and the well-known carcinogenic properties of asbestos, it is my opinion to a reasonable degree of scientific certainty that asbestos exposure can cause ovarian cancer. Even disregarding the evidence that cosmetic talc is contaminated with asbestos, it is my opinion that talc is causally associated with ovarian cancer. However, to the extent that talcum powder products contain even small amounts of asbestos, the evidence of causation is even more compelling.

XVI. BRADFORD HILL ANALYSIS:

In 1965, Sir Austin Bradford Hill proposed nine viewpoints of a causal relationship: strength of association, consistency, specificity, temporality, biologic gradient, plausibility, coherence, experiment and analogy (Hill 1965). It is important to remember, however, as discussed at the beginning of this report, that Hill himself noted that none of these viewpoints of association – including the existence of a statistically significant relationship – is either necessary or sufficient to show causation. There are no "hard-and-fast rules". Rather, the totality of the evidence must be weighed and considered. With that important command in mind, let us examine the evidence.

1. Strength of association:

Strength of association is often measured by the magnitude of the relative risk (CDC). All meta-analyses and pooled analyses have found a statistically significant increased risk of ovarian cancer in perineal talc users, with relative risks falling between 1 and 2. This is consistent with a causal relationship. Strength of association is higher for asbestos. There are a number of examples of causal relationships where the relative risk is less than 2.0 (e.g., second hand smoke and lung cancer, oral contraceptive use and breast cancer, radon exposure and lung cancer). It also is worth noting that small or moderate effects on the benefit side can have important clinical significance. For example, aspirin has been deemed "causal" of cardiovascular event reduction, based on multiple studies that reported a benefit between 20-30% reduction in cardiovascular events. The strength of this association, especially combined

with the consistency, weigh in favor of a cause-and-effect relationship between talc and ovarian cancer.

2. Consistency:

The statistically significant increased risk of ovarian cancer with talc use has been consistent in size across multiple studies, different populations, different investigators, multiple countries and over time. Hill stressed the importance of repetitive findings; no single study can prove or disprove causation due to possible inherent internal validity issues. The consistency of the increased risk of ovarian cancer (and in particular invasive serous carcinoma) with talc use found in numerous studies, in different countries, and after adjustments for confounding factors cannot be disregarded. There also is consistent evidence of an association between asbestos and ovarian cancer. This was a very important factor in my analysis.

3. Specificity:

Hill suggested that associations are more likely to be causal when they are specific, in other words, a particular substance causes a single disease. However, in the half-century experience has shown that this aspect of causation is not particularly important in the context of cancer. Few examples of specificity are found when it comes to cancer. Smoking is generally accepted to be a cause of lung cancer, yet smoking is also associated with COPD, heart disease, stroke, and asthma, amongst other diseases. In multiple studies, talc has been shown to be associated with epithelial ovarian cancer, with invasive serous ovarian cancer showing the strongest association. Asbestos is generally accepted to cause mesothelioma, lung cancer, and ovarian cancer. Asbestos is also generally accepted to cause asbestosis/pulmonary fibrosis, pleural inflammation and thickening. This was a less important factor in my analysis.

4. Temporality:

Exposure to a substance must precede onset of disease for it to be causal. The above-described case-control and cohort studies had the objective of assessing talc exposure that preceded the onset of disease. In cohort studies, the exposure data was obtained before any women were diagnosed with ovarian cancer. In the case-control studies, women with ovarian cancer reported exposures prior to their diagnosis and controls reported exposures in the same time frame. In many studies the exposures went back several decades, providing even more assurance that the temporality requirement is met. This was an important factor in my analysis.

5. Biological gradient:

A biologic gradient, or dose-response, refers to an increased exposure corresponding to an increased risk. In the case of talc exposure, dose-response would ideally include both frequency of use and duration of use, or “application years” (total lifetime applications) similar to “pack-years” used in the setting of smoking. However, application-years is much more difficult to assess than pack-years, since one cannot easily quantify the amount of talc

used during each perineal application (unlike in smoking, where one can easily count the number of cigarettes smoked to calculate pack-years). Yet, when studies have evaluated duration and frequency of perineal talc use, most have found an increased risk of ovarian cancer with increased exposure (Harlow 1992, Cramer 1999, Mills 2004, Merritt 2008, Wu 2009, Terry 2013, Penninkilampi 2018). In the case of asbestos and mesothelioma, a study published by Plato et al. in 2018 found "a significant, dose–response relationship between maximum intensity asbestos exposure and mesothelioma of the pleura and cumulative asbestos exposure with 30-, 40-, and 50-years lag time. Cumulative exposure to asbestos, even at low levels, entailed an increased risk of mesothelioma of the pleura, indicating that even short periods with cumulative doses <1.78 f-y/ml can increase the risk of mesothelioma. Time since first exposure did not show any sufficient dose–response relationship in the longest lag period (>50 years)." (Plato 2018)

While there is evidence of a dose response, this data is more equivocal because of the challenge in measuring and comparing the extent of talcum powder usage. The evidence of biological gradient for talcum powder products is therefore very difficult to study. The evidence of biological gradient supports cause and effect, but for the reasons noted, it is limited by difficulties in the measurement of exposure. This was an important factor in my analysis.

6. Plausibility:

In this context, plausibility means that an association can be explained by and is consistent with existing scientific knowledge and, in particular, that there is a biologically plausible explanation for the exposure (to talc) as a cause of ovarian cancer. Thus, plausibility is dependent upon the current state of scientific knowledge regarding a mechanism of disease. Hill noted plausibility is helpful but limited by current knowledge.

There is evidence that validates the biological plausibility of talc-related ovarian cancer. It is generally accepted that inflammation plays a role in carcinogenesis. Pelvic inflammatory disease and endometriosis are known risk factors for ovarian cancer, and they cause the release of inflammatory mediators. Talc is known to produce an inflammatory reaction, and is in fact used in clinical practice to induce inflammation in the pleura to treat patients with pneumothorax and pleural effusions. It has also been demonstrated that particles, including talc, can migrate proximally through the female genital tract and gain access to the perineum, ovaries, and fallopian tubes. Thus, it is plausible that talc can reach the ovaries and fallopian tubes and cause a proinflammatory reaction, including induction of cytokines and ROS that play a role in the onset of ovarian cancer. Other plausible mechanisms include a down-regulated immune response to MUC1, causing an immune tolerance of a MUC1-expressing cancer, and talc-induced macrophage TNF-α expression and subsequent ovarian tumorigenesis. The 2008 Gates study showed an association of talc and ovarian cancer in women with the GSTT1-null genotype (p-interaction=0.03), particularly in combination with the GSTM1-present genotype (p-interaction=0.03). It is thus plausible that women with a GSST1-null phenotype are unable to eliminate talc as efficiently and are at increased risk of ovarian cancer. It is also highly plausible that asbestos in asbestos-tainted talc also releases cytokines and mutagenic ROS from inflammatory cells.

In the case of asbestos, fiber surface reactivity with free radical generation has been accepted as a mechanism of carcinogenesis (IARC Monograph). Asbestos-derived free radicals can lead to a variety of effects on cells including lipid peroxidation, DNA oxidation, TNF release, cell apoptosis, and increased uptake of asbestos fibers (Mossman 1983, Hobson 1990, Ghio 1998, Churg 1998, Gulumian 1999, Aust 1999, Upadhyay 2003, IARC Monograph). Asbestos fibers may directly cause the generation of ROS (IOM 2006) and indirectly cause ROS by inducing inflammation and macrophage activation (IARC Monograph). As noted above, the carcinogenicity of the other constituents of talc (cobalt, chromium, nickel, and fragrance ingredients) adds strength to biologic plausibility.

This biologic evidence, provides a biologically plausible explanation for the increased risk seen in the epidemiologic studies and is therefore a very strong factor in favor of a cause and effect relationship.

7. Coherence:

Coherence in this context means coherence between epidemiological and generally accepted knowledge of the disease in question. Numerous studies addressing talc use and ovarian cancer have indicated talc use increases ovarian cancer risk consistently. The coherence of the epidemiological evidence linking a risk of ovarian cancer with talc use, in tandem with biologically plausible mechanistic evidence discussed above, is striking and weighs heavily in support of causation.

8. Experiment:

Hill suggested that evidence drawn from experimental manipulation, particularly epidemiologic studies in which disease risk declines following an intervention or cessation of exposure, may lead to the strongest support for causal association. No studies exist that follow women after cessation of genital powder use and assess them specifically for a change in risk of ovarian cancer. The challenge of such a study is that it has been shown that talc-associated ovarian cancer takes years or decades before onset of disease. However, the Australian study performed by The Survey of Women's Health Study Group published in 1997 found that the risk of ovarian cancer was highest among women who were talc users and had not undergone surgical sterilization (RR=1.3, CI 1.1-1.6). (Green 1997). This indicates that tubal ligation or hysterectomy, by impeding the proximal migration of talc into the perineum to the ovaries and fallopian tubes, decreases the risk of talc-associated ovarian cancer, lending support to Hill's experiment aspect in the context of talc and ovarian cancer.

There are experimental studies in the literature that support a causal relationship between talc and ovarian cancer. Examples include studies that show increases in inflammatory markers following talc exposure (Allaire 1989, Genofre 2009, Arellano-Orden 2013). There is also evidence that talc causes neoplastic transformation in ovarian cells (Buz'Zard 2007) and that talc induces genotoxicity in mesothelial cells (Shukla 2009). Additionally, there is evidence that talc induces macrophage TNF-α expression (Cheng 2000), and macrophages that express TNF-α have been shown to promote ovarian tumorigenesis

(Hagemann 2006). Of note, invasive serous carcinomas commonly have p53 mutations and TNF-α induced chromosomal mutations have been shown to occur mostly in cells with p53 aberrations (Yan 2006).

It has long been generally accepted that asbestos exposure causes mesothelioma, ovarian cancer, and lung cancer (Dement 1994, deKlerk 1996, Berry 2000, IARC 2012). The experimental evidence was very important to my analysis.

9. Analogy:

Comparisons of similar associations can be used to determine plausibility. Hill suggested that when there is strong evidence of a causal relationship between a particular agent and a specific disease, researchers should be more accepting of weaker evidence that a similar agent may cause a similar disease. Analogy under the Bradford Hill viewpoints has been interpreted to mean that when one causal agent is known, the standards of evidence are lowered for a second causal agent that is similar in some way (Susser 1991). In the case of talc and ovarian cancer, one can use the analogy of asbestos and mesothelioma. Both talc and asbestos are silicates, and asbestos causes an inflammatory and fibrosing reaction within the pleura, which is generally accepted to be the primary cause of mesothelioma years later. It is the inflammatory and fibrosing reaction caused by talc that has led to its common use in the treatment of pneumothorax and pleural effusions by injection into the pleural cavity. Similarly, in the case of asbestos, fiber surface reactivity with free radical generation has been accepted as a mechanism of carcinogenesis (IARC Monograph). The analogy evidence was somewhat important in my analysis.

XVII. CONCLUSION:

Based upon the totality of the evidence and consideration of the Bradford Hill viewpoints, which includes the high consistency and replication of the findings in the epidemiological studies, pathological, biological, and mechanistic evidence, it is my opinion, which I hold to a reasonable degree of scientific and medical certainty, that genital talcum powder exposure can cause ovarian cancer.

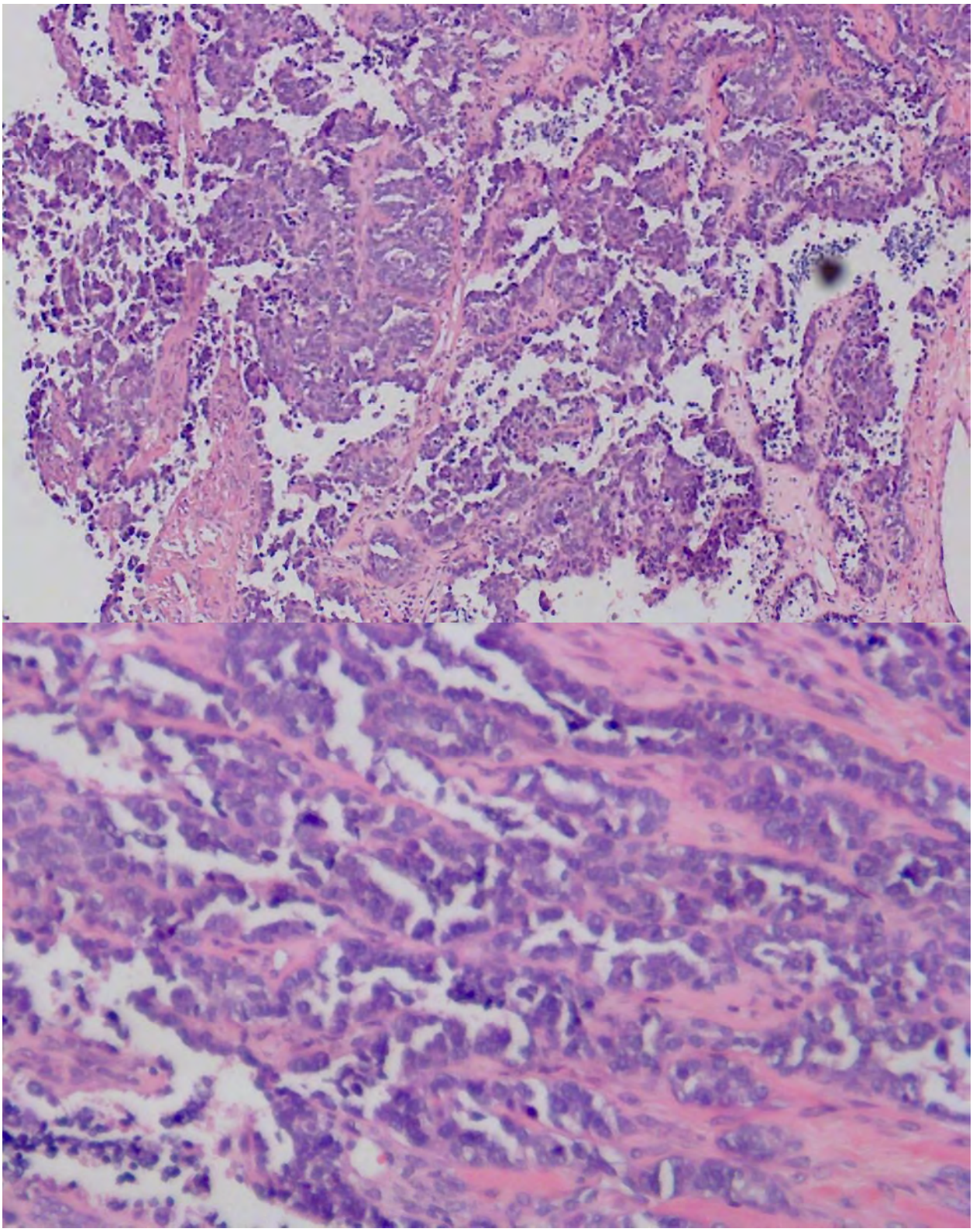


Figure 1. Ovarian invasive serous carcinoma.

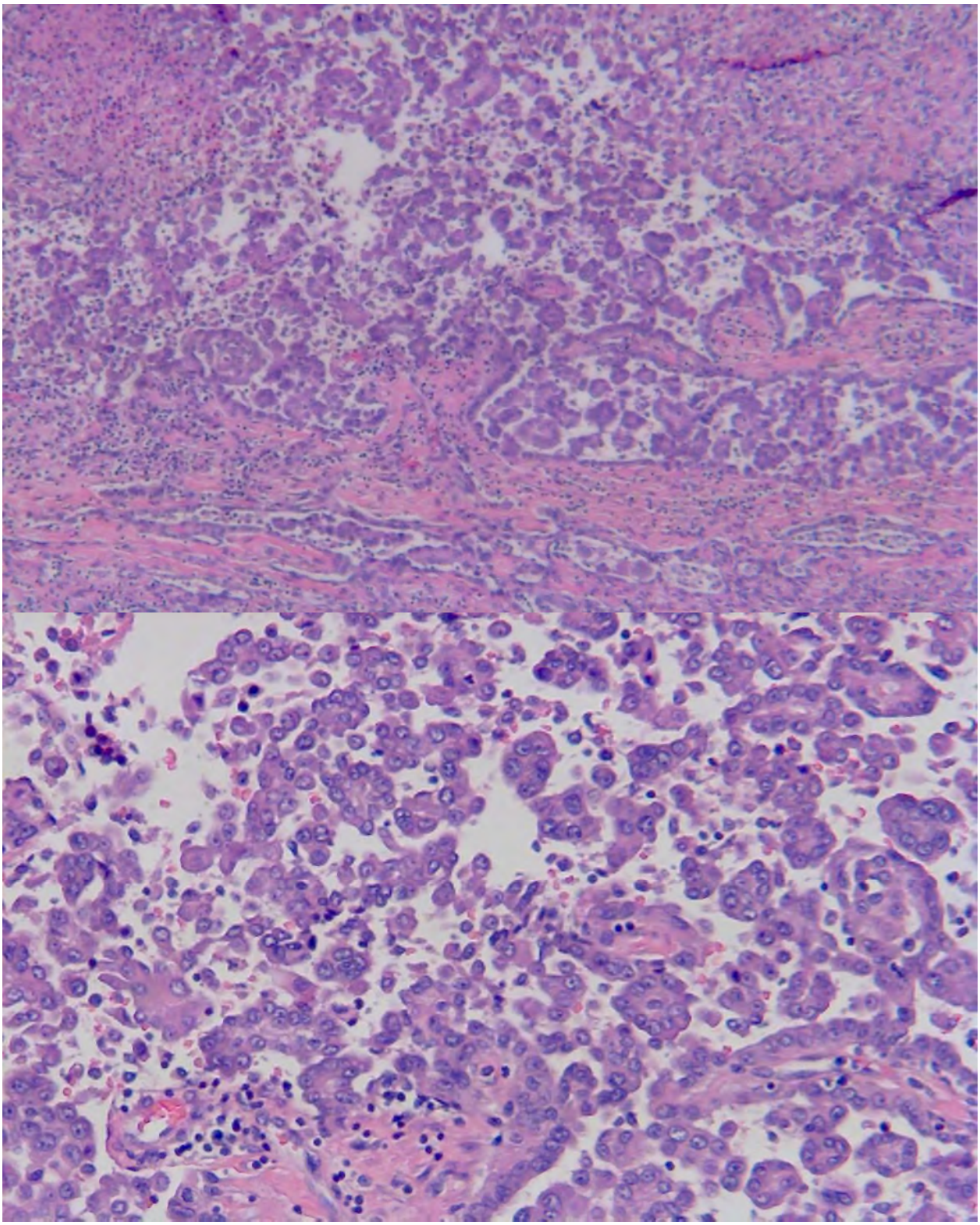


Figure 2. Mesothelioma. Notice the morphologic similarities to ovarian serous carcinoma (Fig 1).

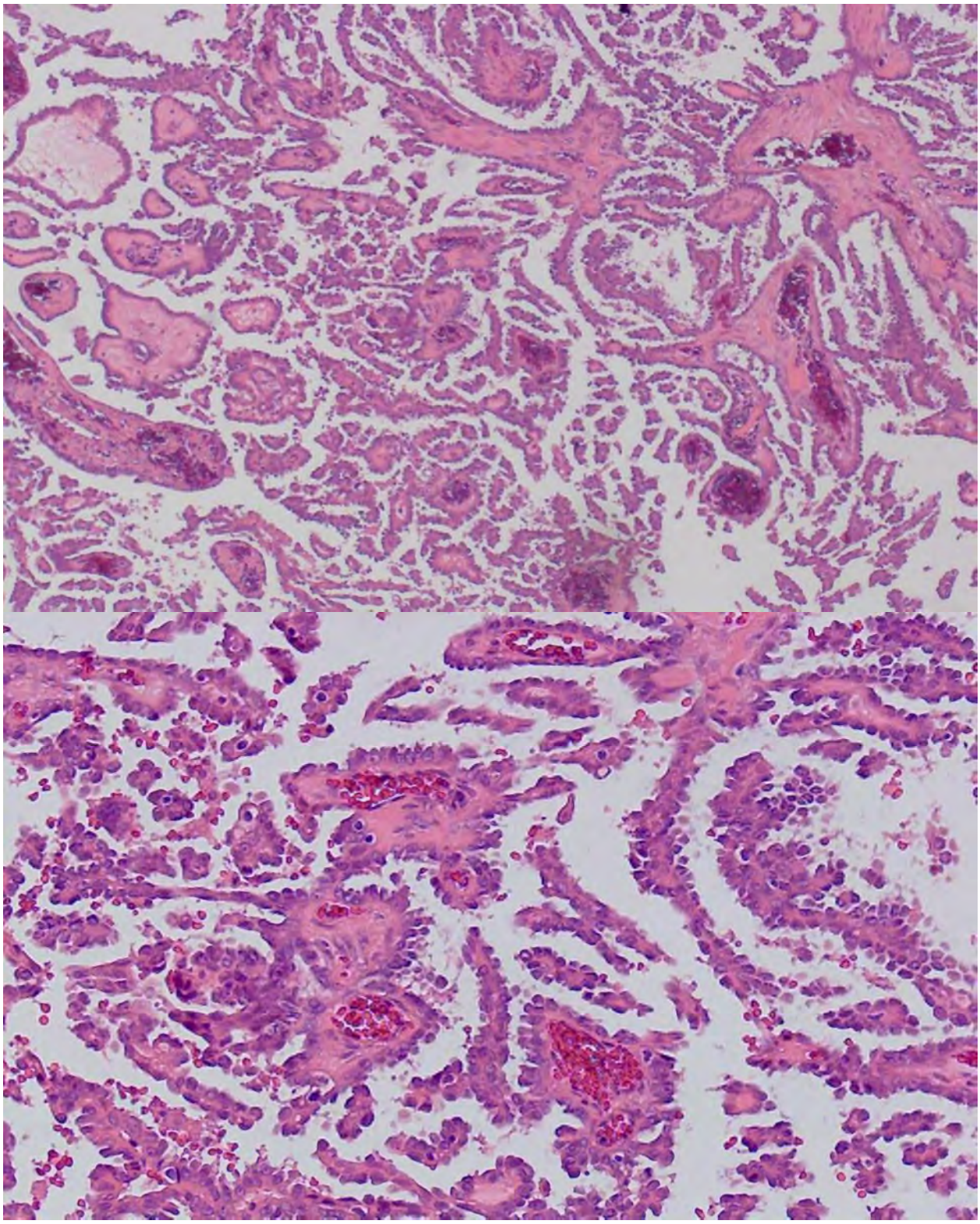


Figure 3. Ovarian serous borderline tumor.

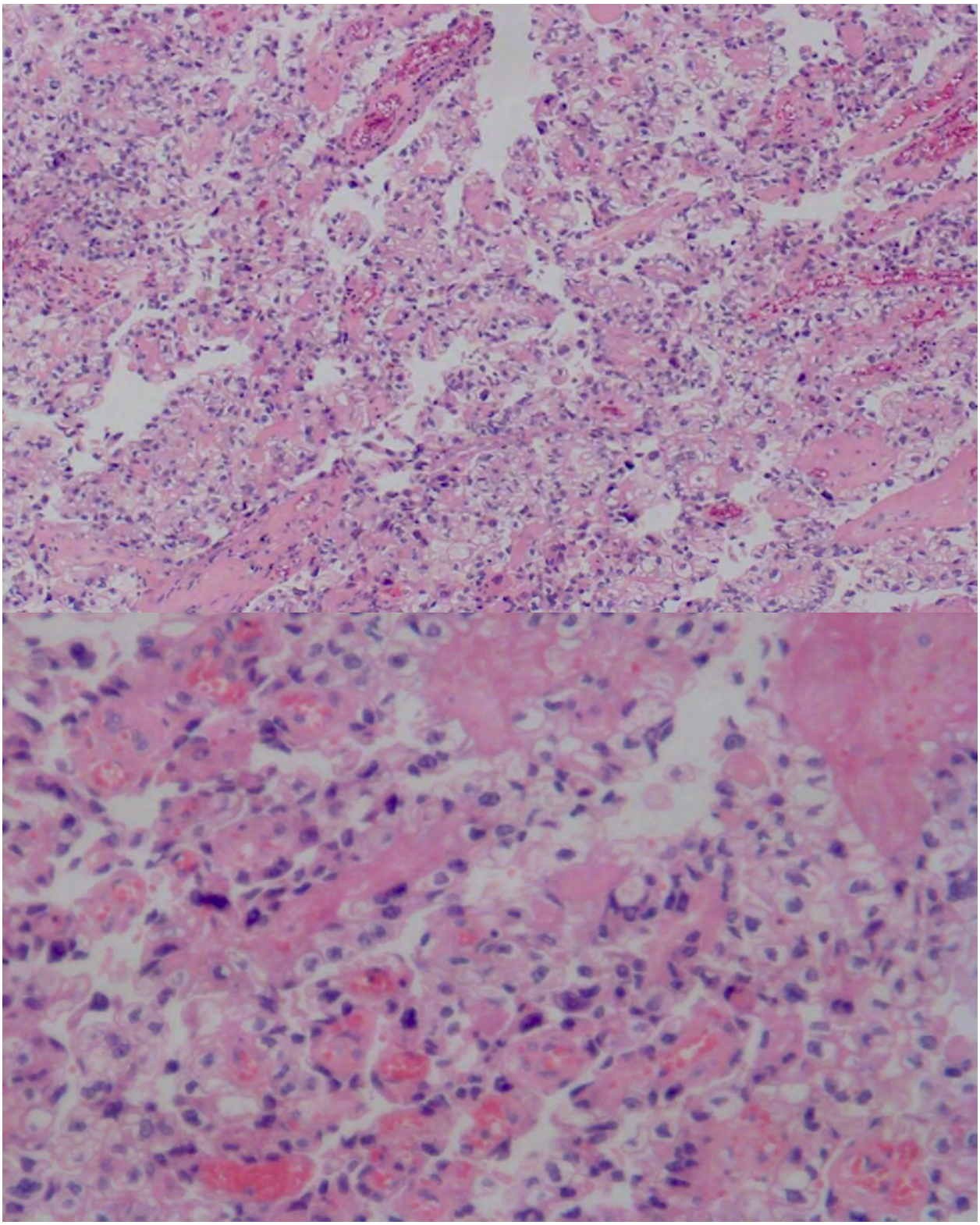


Figure 4. Ovarian clear cell carcinoma.

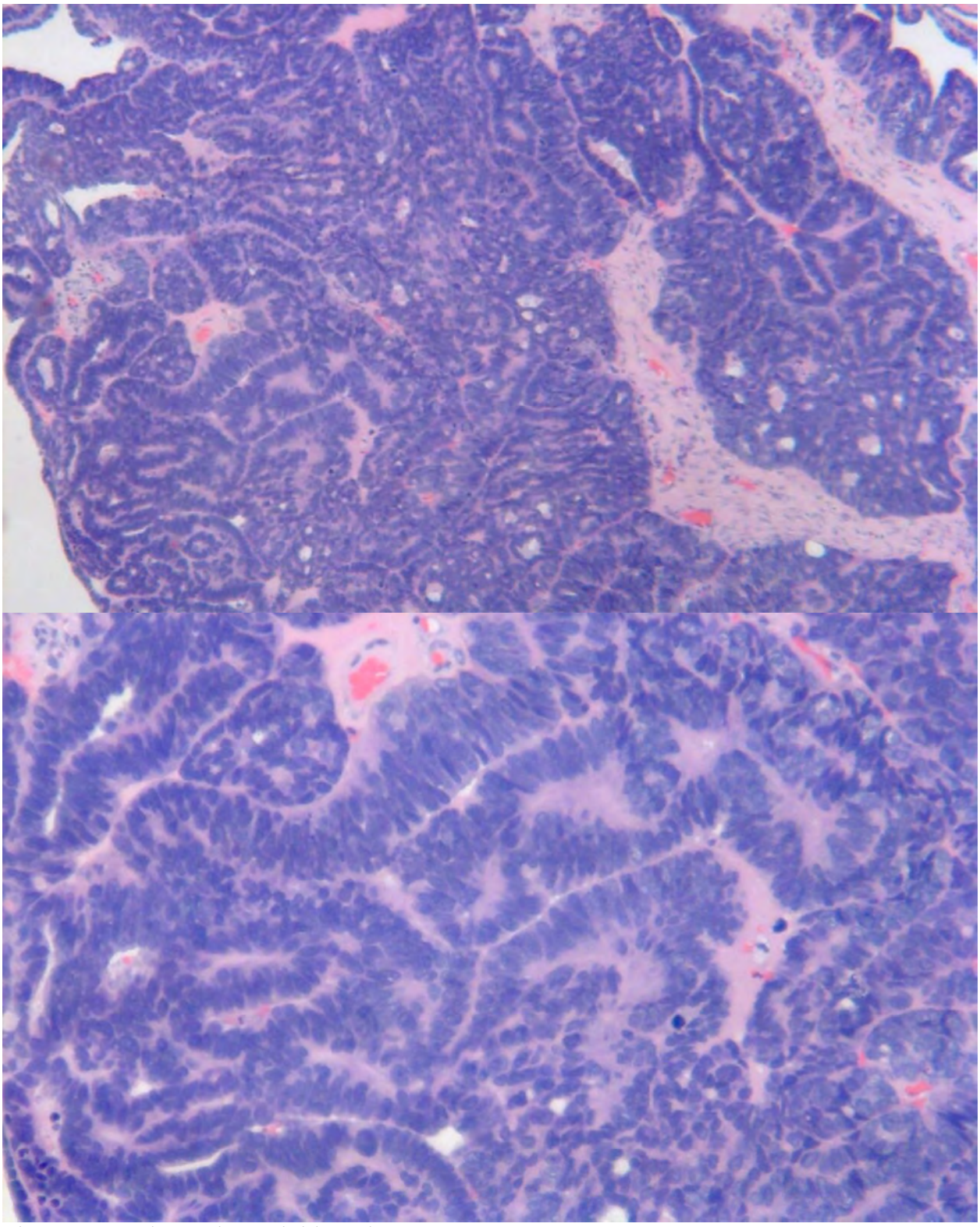


Figure 5. Ovarian endometrioid carcinoma.

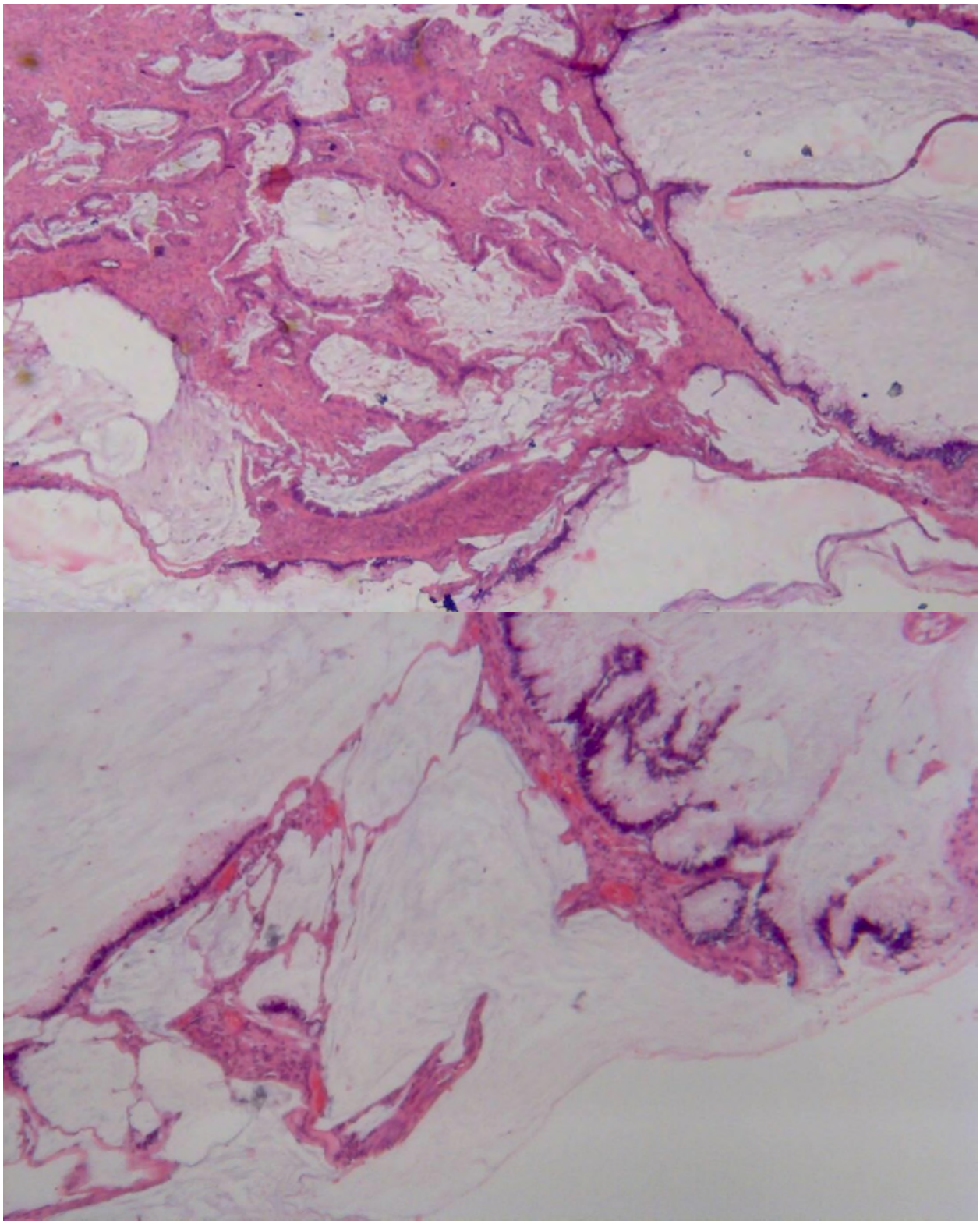


Figure 6. Ovarian mucinous carcinoma.

EXHIBIT A

CURRICULUM VITAE

Date prepared: January 2018

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Place of Birth: Norwalk, CT

Education:

1995	B.A.	Skidmore College Cum laude
2001	M.D.	The Ohio State University College of Medicine

Postdoctoral Training:

2001-2005	Resident	Pathology, AP/CP	Massachusetts General Hospital
2005-2007	Fellow	Robert E. Scully Fellow Cytopathology, Gynecologic and Perinatal Pathology	Massachusetts General Hospital

Academic Appointments:

2001-2005	Clinical Instructor	Pathology	Harvard Medical School
2005-2007	Graduate Assistant	Pathology	Harvard Medical School
2007-2011	Instructor	Pathology	Harvard Medical School

Appointments at Hospitals/Affiliated Institutions

2007-2011	Staff Pathologist	Pathology	Beth Israel Deaconess
2007-2011	Staff Pathologist	Pathology	Beth Israel Deaconess-Needham
2011-Present	Staff Pathologist	Pathology	North Shore Medical Center
2011-Present	Staff Pathologist	Pathology	Newton-Wellesley Hospital
2011-Present	Clinical Affiliate	Pathology	Massachusetts General Hospital

Major Administrative Responsibilities:

2005	Chief Resident, Anatomic Pathology	Massachusetts General Hospital
2007-2011	Course Director, PA501.5 Elective	Harvard Medical School
2010-2011	Associate Director, Cytopathology Fellowship	BIDMC/Harvard
2012-2013	Hematology Laboratory Director NSMC	NSMC/Partners
2013-Present	Autopsy Director, North Shore Medical Center	NSMC/Partners

Major Committee Assignments:

2005-2007	Cytopathology	Junior Member	College of American Pathologists
2005	Path Residency Training Committee	Member	Massachusetts General Hospital
2005	Anatomic Path Quality Assurance	Member	Massachusetts General Hospital
2005	Anatomic Path Steering Committee	Member	Massachusetts General Hospital
2008-2011	Path Resident Selection Committee	Member	Beth Israel Deaconess
2009-2011	Path Residency Planning Committee	Member	Beth Israel Deaconess
2010	Pathology Scheduling Committee	Member	Beth Israel Deaconess
2010-2011	Anatomic Path Quality Assurance	Member	Beth Israel Deaconess

Professional Societies:

1997 – 2001	American Medical Student Association	Member
2001 – Present	Massachusetts Medical Society	Member
2003 – Present	United States and Canadian Academy of Pathology	Member
2005 - Present	College of American Pathologists	Member

Awards and Honors:

1994	Charlotte W. Fahey Prize in Chemistry, Skidmore College
1994	Skidmore College Periclean Honor Society
1995	Phi Beta Kappa, Skidmore College
1995	Cum Laude with Department Honors, Skidmore College
2000	Honors in Pediatric Hematology and Oncology 4th Year Clerkship
2000	Letter of Commendation, Surgery Third Year Clerkship
2000	Letter of Commendation, Neurology Third Year Clerkship
2001	Honors in Anatomic and Clinical Pathology Fourth Year Elective
2001	Honors in Individual Studies in Pathology Fourth Year Elective
2016	Partners in Excellence Team Award

Teaching of Students:

Harvard Medical School Courses:

2007-2009	Respiratory Pathophysiology	
2nd Year Medical Students	Lab Instructor	Three 2 hour sessions, one week

2007-2009	Cardiovascular Pathophysiology	
2nd Year Medical Students	Lab Instructor	Three 2 hour sessions, one week
2007-2011	Core Surgery Clerkship	
3rd Year Medical Students	Pathology Coordinator	One hour lecture/3 months
2009-2011	Principal Clinical Experience	
3rd Year Medical Students	Mentor	Two hour session per week
2009-2011	Principal Clinical Experience – Pathology Elective	
3rd Year Medical Students	Mentor	Minimum 2 hour session/month

Formal Teaching of Residents:

2007	Respiratory Cytology	
All pathology residents	Beth Israel Deaconess	One hour lecture
2007-2011	Respiratory Cytology	Quarterly 1 hr microscope session
Pathology residents rotating through Cytology		
2008-2011	Fine Needle Aspiration Techniques	
All pathology residents	Beth Israel Deaconess	One hour lecture
2008-2011	Histologic and Cytologic Correlation of Cervical Lesions	
All pathology residents	Beth Israel Deaconess	One hour lecture

Clinical Supervisory and Training Responsibilities:

2007-2011 Core Surgery Clerkship, Pathology Elective BIDMC 2 students/month

Local Invited Presentations:

2005 Cytology/Histology Correlation Clinical Pathology Technician Lecture Series
Department of Pathology, Massachusetts General Hospital

2008 Respiratory Cytology Cytopathology Lecture Series
Department of Pathology, Brigham and Women's Hospital

Current Licensure and Certification:

2005 Full License, Massachusetts

2008 Board certified, Anatomic and Clinical Pathology

2008 Board certified, Cytopathology

Practice Activities:	
Surgical Pathology, Cytopathology, Autopsy	North Shore Medical Center
Surgical Pathology, Cytopathology	MGH Ambulatory Care Center
Cytopathology	Massachusetts General Hospital
Clinical Pathology	Newton-Wellesley Hospital

Peer-Reviewed Publications:

Narasimhan V, Malboueuf B, **Hodil SE**. Temperature Induced Interstrand Crosslinks in Cisplatin-DNA Adducts Detected by Electrophoresis and UV Spectrophotometer. *Biochem Mol Biol Int*. 1995;37:843-851.

Grundy FJ, Hodil SE, **Rollins SM**, Henkin TM. Specificity of tRNA-mRNA Interactions in *Bacillus subtilis* tyrS antitermination. *J Bacteriol*. 1997;179:2587-2594.

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Chan MP, Hecht JL, **Kane SE**. Clinicopathologic Correlation of Fetal Vessel Thrombosis in Mono- and Dichorionic Twin Placentas. *J Perinatol*. 2010 Oct; 30(10):660-4.

Kane SE, Hecht JL. Endometrial Intraepithelial Neoplasia Terminology in Practice: 4-Year Experience at a Single Institution. *Int J Gynecol Cancer*. 2012 Mar;31(2):160-165.

Haspel RA, Bhargava P, Gilmore H, **Kane SE**, Powers A, Sepehr A, Weinstein A, Schwartzstein R, Roberts D. Successful Implementation of a Longitudinal, Intergrated Pathology Curriculum During the Third Year of Medical School. *Arch Pathol Lab Med*. 2012 Nov;136(11):1430-6.

Proceedings of Meetings (Poster Presentations):

Rollins S, Prayson RA, McMahon JT, Cohen BH. Diagnostic Yield of Muscle Biopsy in Patients With Clinical Evidence of Mitochondrial Cytopathy. 90th United States and Canadian Academy of Pathology. March 2001. Atlanta, GA.

Rollins SE, Nielsen GP, Hedley-Whyte ET. Light Microscopy, Electron Microscopy, and Mitochondrial Enzyme Function in Muscle Biopsies for Suspected Mitochondrial Cytopathies. 92nd United States and Canadian Academy of Pathology. March 2003. Washington, DC.

Rollins SE, Nielsen GP, Hedley-Whyte ET. Light Microscopy, Electron Microscopy, and Mitochondrial Enzyme Function in Muscle Biopsies for Suspected Mitochondrial Cytopathies. Massachusetts General Hospital Clinical Research Day. June 2003. Boston, MA.

Rollins SE, Young RH, Bell DA. Autoimplants Involving Serous Borderline Tumors of the Ovary: A Clinicopathologic Study of 30 Cases. 93rd United States and Canadian Academy of Pathology. March 2004. Vancouver, BC.

Michaels PJ, **Rollins SE**, Bounds BC, Brugge WR, Pitman MB. Cyst Fluid Analysis and Endoscopic Features Aid in the Preoperative Grading of Intraductal Papillary Mucinous Neoplasms of the Pancreas. 95th United States and Canadian Academy of Pathology. February 2006. Atlanta, GA.

Rollins SE, Clement PB, Young RH. Uterine Tumors Resembling Ovarian Sex Cord Tumors Frequently Have Incorporated Mature Smooth Muscle Imparting a Pseudoinfiltrative Appearance. 96th United States and Canadian Academy of Pathology, March 2007. San Diego, CA.

White SR, Hecht J, **Kane SE**, Fu Y, Cohen DW, Wang HH. Bile duct brush cytology: indeterminate diagnosis is essential. Arch Pathol Lab Med 2009;133:1689.

EXHIBIT B

SARAH E. KANE, M.D.

Board Certified in Anatomic and Clinical Pathology, and Cytopathology

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AND OTHER MATERIAL AND DATA CONSIDERED

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Exhibit 49

AMERICAN JOURNAL OF INDUSTRIAL MEDICINE 29:435–439 (1996)

Asbestos Exposure and Ovarian Fiber Burden

Debra S. Heller, MD, Ronald E. Gordon, PhD, Carolyn Westhoff, MD, and Susan Gerber, MD

Epidemiologic studies suggest increased risk of epithelial ovarian cancer in female asbestos workers and increased risk of malignancy in general in household contacts of asbestos workers. Ovaries were studied from 13 women with household contact with men with documented asbestos exposure and from 17 women undergoing incidental oophorectomy. Ovarian tissue was examined by analytic electron microscopy.

Significant asbestos fiber burdens were detected in 9 out of 13 women with household asbestos exposure (69.2%), and in 6 out of 17 women who gave no exposure history (35%). Three exposed women had asbestos counts over 1 million fibers per gram wet weight (23%), but only 1/17 women without an exposure history had a count that high (6%). Although asbestos has been documented as a contaminant of some older cosmetic talc preparations, the chrysotile and crocidolite types of asbestos we detected are more indicative of background and/or occupational exposure.

This study demonstrates that asbestos can reach the ovary. Although the number of subjects is small, asbestos appears to be present in ovarian tissue more frequently and in higher amounts in women with a documentable exposure history.

KEY WORDS: asbestos, ovary, talc, environmental exposure

INTRODUCTION

Epidemiologic evidence suggests that there is an increased risk of ovarian carcinoma in female asbestos workers [Acheson et al., 1982; Graham and Graham, 1967; Keal, 1960; Newhouse et al., 1972, 1985; Wignall and Fox, 1982], and animal data show changes resembling early ovarian carcinoma after intraperitoneal injection of asbestos [Graham and Graham, 1967]. In addition, household contacts of asbestos workers have been shown to be at increased risk of developing asbestos-related disease [Joubert et al., 1991; Roggli and Longo, 1991], so female household contacts of asbestos workers may also be at risk of ovarian exposure to asbestos. There is literature that supports that perineal talc exposure increases the risk of ovarian carcinoma. However, some of these data have been clouded by the fact that cosmetic talc was often contaminated with asbestos in the past, particularly before 1976 [Cramer et al., 1982]. Particulate matter can reach the female peritoneal cavity via the transvaginal route [Egli and Newton, 1961; Henderson et al., 1986; Joubert et al., 1991]. A woman exposed to her husband's occupationally contaminated laundry may have asbestos enter the peritoneal cavity by passive transfer, or even by sexual relations. The purpose of this study was to determine whether women exposed to asbestos have a high asbestos fiber burden in their ovaries.

MATERIALS AND METHODS

Eligible women were contacted by postcard with the assistance of a law firm specializing in asbestos-related claims. Women with household contact to asbestos, as documented by interview, and who had themselves previously undergone ovarian surgery, were invited to participate. No women with direct occupational exposure responded.

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TABLE I. Demographics and Pathologic Findings Among 8 Patients With Household Contacts Evaluated for Asbestos-Related Disease Oophorectomized Between 1973–1994, and 5 Oophorectomized Patients With a History of Asbestos Exposure From the Columbia Presbyterian Medical Center Benign Neoplasm Study, 1992–1993

Subject	Reason for surgery	Asbestos-fibers per gram wet weight	Limits of detection	Asbestos exposure
1	Papillary serous cystadenocarcinoma of ovary	490,813 chrysotile: crocidolite 1:2	40,901	Husband—pipefitter with asbestos
2	Mucinous cystadeno-carcinoma of ovary	below detectible limits	26,267	Father—died of mesothelioma; husband—asbestosis (both insulators)
3	Endometrial adenocarcinoma	1,227,031 chrysotile: crocidolite 1:1	15,338	Husband—asbestosis, carpenter in a factory
4	Atypical hyperplasia of endometrium	74,167 chrysotile	18,542	Husband—insulator, died of lung cancer
5	Endometriosis of ovary	328,913 chrysotile	41,114	Father and aunt—worked in asbestos plant; father—died of lung cancer, aunt of asbestosis
6	Leiomyoma uteri	3,438,636 chrysotile	42,983	Father—asbestosis, asbestos and insulation worker
7	Serous cystadenoma of ovary, fibroma of ovary	below detectible limits	42,983	Husband—insulation worker
8	Endometrial adenocarcinoma	1,513,00 chrysotile: tremolite 4:1	37,825	Husband—died of asbestosis;[a] worked as carpenter and with concrete
9[a]	Cystadenofibroma of ovary	49,081 chrysotile: crocidolite 1:1	24,541	2 brothers—construction workers
10[a]	Benign epithelial cyst of ovary	below detectible limits	37,825	Father—shipyard worker and school engineer
11[a]	Serous cystadenoma of ovary	298,618 chrysotile	24,885	Household member—shipyard worker × 4 years
12[a]	Cystadenofibroma of ovary	788,020 crocidolite	157,604	Father—shipyard worker
13[a]	Cystadenofibroma of ovary	below detectible limits	42,983	Household contact —construction/insulation × 3 years

[a]Subjects from Columbia Presbyterian Medical Center Benign Neoplasm Study.

Women with both benign and malignant disease responded. Women undergoing oophorectomy for benign ovarian neoplasms at Columbia Presbyterian Medical Center who were interviewed in depth for another study were available as controls and were included after ascertaining asbestos exposure history and availability of nonneoplastic ovarian tissue for analysis. These women were chosen for the availability of interviews as well as tissue. Five of these women were found to have sustained asbestos exposure. There were 13 exposed subjects and 17 women who gave no history of exposure. Tissue from two stillborn ovaries was also analyzed. Tissue blocks of benign adjacent or contralateral ovarian tissue as available were obtained, and analytic electron microscopy was performed according to the subsequent protocol. Hematoxylin and eosin stained sections of analyzed tissue were examined. There was no evidence of response to asbestos such as foreign body giant cell reactions or fibrosis in the tissue. Ovarian tissue does not undergo fibrosis as does lung.

Analytic Electron Microscopy Protocol

Ovarian tissue in blocks was deparaffinized, rehydrated, blotted dry, and weighed. Digestion with 5% KOH was performed at 70°C for 2–4 hr. After complete digestion, the tissue was centrifuged at 12,000 rpm for 20 min. The KOH was removed, leaving a pellet to which approximately 20 ml of distilled water was added. The pellet was resuspended by using a microultrasonic cell disrupter at 50 watts for 5 sec. Centrifugation, distilled water wash, and

TABLE II. Demographics and Pathologic Findings Among 17 Oophorectomized Patients With No History of Asbestos Exposure—Columbia Presbyterian Medical Center Benign Neoplasm Study, 1992–1993

Control	Reason for surgery	Asbestos fibers per gram wet weight	Limits of detection	Exposure history
11 subjects	4 serous cystadenomas/simple cyst 3 benign cystic teratoma/struma ovarii 2 endometrioma/endometriosis 1 fibrothecoma 1 mucinous cystadenoma	Below detectable limits	None greater than 27,267	None
1	Endometriosis, benign cystic teratoma	525,871 chrysotile: crocidolite 1:2	17,529	None
2	Endometrioma of ovary	109,069 chrysotile: crocidolite 1:1	6,817	None
3	Benign cystic teratoma of ovary	33,849 chrysotile	8,462	None
4	Endometrioma of ovary	147,244 chrysotile: crocidolite 1:2	12,270	None
5	Serous cystadenoma of ovary	98,163 crocidolite	12,270	None
6	Benign cystic teratoma of ovary	2,181,388 chrysotile	27,267	None

microultrasonic cell disrupter were repeated 3 times. The distilled water was removed and the pellet was resuspended in 5–10 ml of distilled water. Ten-µl drops of the final suspension were placed on nickel Formvar and carbon-coated locator grids and air dried. Transmission electron microscopy to identify fibers, and their size was performed. The identity of the fibers was determined by energy-dispersive spectroscopy and confirmed by electron diffraction (SAED). Grids were viewed at both 10,000 and 19,000 diameters. All fibers observed were counted.

Routinely, as they are opened or distilled water at each filtering, all solutions are checked for detectable limits of asbestos fibers. All places where asbestos could have contaminated the specimen, such as paraffin, are also controlled for paraffin blocks from each different source. All solutions are checked by passing the fluids through a 0.1-µm Nucleopore filter to maximize the efficiency of detecting and counting fibers present in these solutions and materials. The solutions that are routinely tested are distilled water, KOH, and xylene. If detectable levels of asbestos fibers exist in the solutions used to initially fix and process the tissue because they came from different hospitals, at which it was not possible to test these solutions directly, they would be detected in the paraffin controls. We have yet to identify detectable levels in any of the solutions or paraffin.

RESULTS

A summary of the results can be seen in Tables I and II. Nine of the 13 women exposed to asbestos had asbestos in their ovarian tissue (69.23%), with 3 (23%) of them having counts over 1 million fibers per gram of wet weight. Among the controls, 6/17 women had detectable asbestos in their ovaries (35%), with only 1 (6%) patient with a count over 1 million fibers per gram wet weight. In addition, talc was detected in 11/13 exposed women (85%) and in all 17 controls (100%). No asbestos or talc was detected in the stillborn material.

All fibers were counted and analyzed for type and size. The results of that analysis are summarized in Table III. In general, the fibers were relatively small with regard to length and narrow in diameter. However, the great majority of fibers were greater than 3 µm with a minimum aspect ratio of 10. Except for one case, in which tremolite was observed, the fibers were either chrysotile or crocidolite, or both.

DISCUSSION

Epithelial ovarian carcinoma is a major cause of female mortality [Greene et al., 1984]. Epithelial ovarian cancer

TABLE III. Asbestos Fibers in Ovarian Tissues: Type, Number, and Dimensions

Subject	No. of fibers	Fiber type	<3 μm long	3–10 μm long	>10 μm long	<0.1-μm diameter	0.1–0.2-μm diameter	>0.2-μm diameter
1[a]	4	Chrysotile	1	2	1	4	—	—
	8	Crocidolite	1	7	—	4	4	—
3[a]	40	Chrysotile	2	28	10	35	5	—
	40	Crocidolite	3	31	6	30	10	—
4[a]	4	Chrysotile	—	3	1	4	—	—
5[a]	8	Chrysotile	1	6	1	7	1	—
6[a]	80	Chrysotile	5	62	13	71	9	—
8[a]	32	Chrysotile	2	22	8	22	10	—
	8	Tremolite	1	7	—	—	6	2
9[a]	1	Chrysotile	—	1	—	1	—	—
	1	Crocidolite	—	1	—	1	—	—
11[a]	12	Chrysotile	1	9	2	8	4	—
12[a]	20	Crocidolite	2	14	4	12	8	—
1[b]	10	Chrysotile	1	8	1	4	6	—
	20	Crocidolite	2	18	—	17	3	—
2[b]	8	Chrysotile	—	7	1	5	3	—
	8	Crocidolite	1	7	—	6	2	—
3[b]	4	Chrysotile	—	4	—	3	1	—
4[b]	4	Chrysotile	—	4	—	3	1	—
	8	Crocidolite	1	7	—	7	1	—
5[b]	8	Crocidolite	—	8	—	6	2	—
6[b]	80	Chrysotile	7	58	15	68	12	—

[a]From Table I.
[b]From Table II.

develops from the surface epithelium of the ovary, which is embryologically derived from the same tissue as the mesothelium of the abdominal cavity, the celomic epithelium [Falkson, 1985]. Thus, ovarian carcinoma and malignant mesothelioma of the peritoneal cavity are believed by some to be related neoplasms [Parmely and Woodruff, 1974]. Asbestos causes malignant mesothelioma, and there is evidence to support it as an etiology in ovarian carcinoma as well [Acheson et al., 1982; Falkson, 1985; Graham and Graham, 1967; Keal, 1960; Newhouse, 1979; Newhouse et al., 1972, 1985; Whittemore et al., 1988; Wignall and Fox, 1982].

Intraperitoneal injection of tremolite asbestos into guinea pigs and rabbits was shown to cause epithelial changes in their ovaries similar to those seen in early ovarian cancer [Graham and Graham, 1967]. These investigators also found birefringent crystalline material near these epithelial changes, but no further attempt was made to identify the material. No such material was found in controls. Asbestos fibers have been shown to be cytotoxic to Chinese hamster ovary (CHO) cells, an epithelioid cell culture line [Neugut et al., 1978].

Several investigators have cited an increased mortality from ovarian cancer in female asbestos workers exposed as gas mask assemblers or other factory workers [Acheson et al., 1982; Newhouse, 1979; Newhouse et al., 1972, 1985; Wignall and Fox, 1982]. In addition, it is known that household contacts of asbestos exposed workers are also at increased risk of developing malignant disease in general [Joubert et al., 1991; Roggli and Longo, 1991]. In a study of 52 histologically confirmed malignant mesotheliomas in women, most with no occupational exposure of their own, a significant number were found to have husbands or fathers who worked in an asbestos-related industry [Vianna and Polan, 1978], and the findings suggested indirect exposure to a husband as the most important factor.

The fact that exposure to a husband is more significant than exposure to a father suggests a possible role for sexual contact as a transporting vector for asbestos fibers. Household exposure has been related to the asbestos dust on the workers' clothing, with risk to those who launder the clothing [Joubert et al., 1991]. While this may be the exposure source in wives as well as in daughters, it is possible that sexual contact with a male contaminated with asbestos fibers introduces those fibers into the vagina of his partner, where they can reach the peritoneal cavity. There is evi-

dence of transport of particulate matter into the female peritoneum by the transvaginal route, in both human and animal studies [Egli and Newton, 1961; Henderson et al., 1986; Venter and Iturralde, 1979]. Whittemore et al. [1988] suggested that vaginal exposure to particulate matter such as asbestos and talc was a potential risk factor for intraperitoneal ovarian exposure. Her conclusion was based on finding that in talc-exposed women, a previous history of hysterectomy or tubal ligation, which blocks peritoneal access, was protective against ovarian cancer.

Talc has also been implicated as a possible etiologic agent in ovarian cancer [Harlow et al., 1989, 1992], and this is related to the asbestos problem in several ways. Aside from the chemical similarities between the two, many cosmetic talcs contained significant amounts of asbestos, particularly prior to 1976 [Cramer et al., 1982]. The significance of the detection of talc in the majority of the exposed women and in all women giving no exposure history is unclear, and further studies are under way to further elucidate this association.

CONCLUSIONS

In our study, the women with a positive exposure history had asbestos detected in their ovaries more frequently, and in higher counts. None of the exposed subjects in this study was directly occupationally exposed, but all were passively exposed to a household contact. It is unclear why so many of the women giving no exposure history did have detectable asbestos in their ovaries, although it is known that there is a background level of asbestos in the lung tissue of nonexposed individuals. All our available control patients were selected from a group of extensively interviewed women with benign ovarian neoplasms. Further studies are aimed at women with no ovarian pathology. The significance of the finding of asbestos in ovaries requires further investigation.

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Exhibit 50

Foreign Body Granulomas in Normal Ovaries

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In 100 consecutive cases in which grossly normal ovaries were removed at the time of pelvic surgery, 9% were found to contain crystalline foreign particles. An additional 9% contained cortical granulomas. In four of six cases, computer-assisted x-ray analysis of the crystalline foreign particles was successful and revealed magnesium and silicon. ***(Obstet Gynecol 66:701, 1985)***

To make plausible the suggestion that inorganic particulate matter plays a role in the development of proliferative disorders in the female pelvis,[1-5] it is necessary to demonstrate that such matter is capable of producing proliferations under some circumstances. It is also necessary to demonstrate that particulate matter is actually present in the female pelvis with sufficient frequency to account for the amount of observed disease. The present study was designed to address this second question; it does not address the first. It also seeks to determine the elemental nature of the particles observed.

Materials and Methods

In 100 consecutive cases in which grossly normal ovaries were removed at the time of pelvic surgery for other indications, the entire gonad(s) was submitted for histologic examination. A total of 175 normal ovaries were examined. Two to five paraffin blocks were made from each excised gonad, and an average of three sections per ovary were studied. Findings in these 175 ovaries were divided into four groups: cases in which there were no histologic abnormalities, group 1; cases in which there were laminated calcifications, classically referred to as "psammoma bodies," group 2 (Figure 1); cases in which there were foci of reticular stroma with or without inflammation that have been classically referred to as "cortical granulomas," but have been described as endometriosis by others,[6] group 3 (Figure 2); and cases in which foci similar to those in group 3 appeared and which additionally contained foreign body type giant cells and associated crystalline foreign body, group 4 (Figure 3). If two ovaries were removed from one patient, they were classified together as a single case.

Figure 1. Two laminated focal calcifications occupy papillary fronds in this proliferating pelvic neoplasm.

Six examples of crystalline foreign bodies were then processed for examination by scanning electron mi-

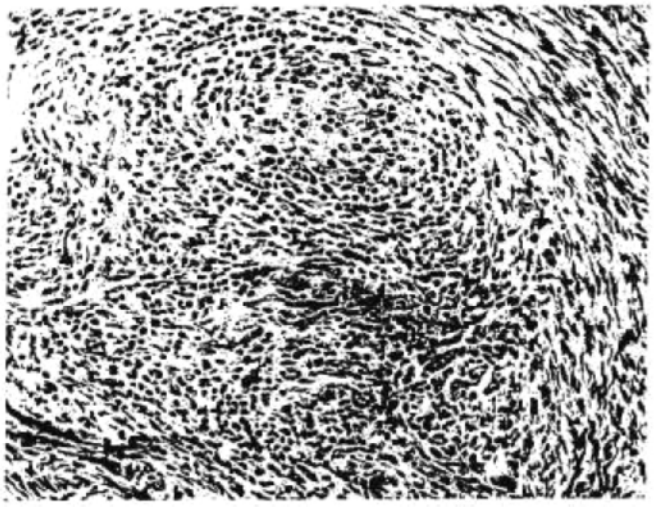

Figure 2. This focus of inflammation was present in the ovarian cortex. Although granulomatous in nature, no giant cells or particulate foreign matter is observed.

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0029-7844/85/$3.30

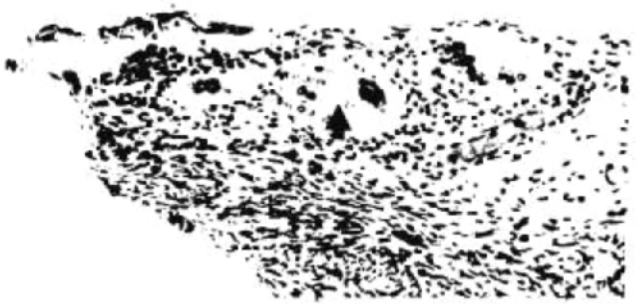

Figure 3. This focus of inflammation in the ovarian cortex contains giant cells. The clefts within the giant cells were filled with refractile crystalline material (*arrows*).

croscopy (ETEC microscope). This consisted of making a number of visible light micrographs in order to record the location of foreign bodies in the specimen and then removing the slide cover slips. The exposed specimens were then mounted on an electron microscope slide and carbon coated. Using appropriate computer-assisted microscopic x-ray analysis, the elemental composition of the crystalline foreign bodies were determined in four cases. In the two other cases, the foreign body was lost when the cover slip was removed.

Results

One hundred seventy-five grossly normal ovaries were removed at the time of pelvic surgery. The surgery was performed for the indications listed in Table 1. Seventy-two cases were classified in group 1 (Table 2).

Computer-assisted x-ray analysis of the crystalline foreign bodies was successful in four of six cases and demonstrated that the particles were composed largely of magnesium and silicon. The mean ages of each group and the percentage with a history of laparotomy also are listed in Table 2.

Table 1. Pelvic Surgery for Various Gynecologic Disorders

Diagnosis	No. of cases
Myomata uteri	42
Endometrial carcinoma	18
Cervical carcinoma	10
Endometrial & cervical carcinoma	1
Mixed mesodermal tumor of cervix	1
Uterine leiomyosarcoma	1
Adenomyosis	3
Parovarian cyst	2
Unilateral ovarian neoplasm	5
Endometrial polyp hyperplasia	1
Salpingitis	4
Pelvic endometriosis	3
Chronic pelvic pain	2
Pelvic inflammatory disease	1
Dysfunctional uterine bleeding	6
Total	100

Table 2. Findings in 100 Consecutive Cases in Which a Grossly Normal Gonad(s) Was Removed

Group	No. of cases	Mean age	% Previous laparotomy
1	72	44	36
2	10	50	30
3	9	52	22
4	9	62	44

Discussion

The most common compounds containing magnesium silicates in industrial North America are talc and asbestos. As reported,[1–3,5] it is not a new observation that talc may be found in the pelvis, nor are talc granulomas in and of themselves new observations. However, the fact that 9% of the women operated on in the Johns Hopkins Hospital for pelvic disease appeared to have magnesium silicate granulomas in their normal ovaries, and that an additional 9% contained histologic entities that were very similar, represents a higher incidence than the authors had suspected. The exact figure is probably not relevant as it, undoubtedly, varies from population to population, depending on the exposure sustained by that given population. Nevertheless, in at least one geographic area, the incidence of foreign body contamination in the pelvis is sufficiently high to account for the incidence in that geographic locale of proliferative disorders seen at that anatomic site.

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Exhibit 51

Presence of Talc in Pelvic Lymph Nodes of a Woman With Ovarian Cancer and Long-Term Genital Exposure to Cosmetic Talc

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BACKGROUND: Although epidemiologic studies suggest talc use may increase ovarian cancer risk, there is no proof that talc used externally reaches the pelvis.

CASE: A 68-year-old woman with stage III ovarian papillary serous carcinoma revealed she had used talc daily for 30 years to powder her genital area. Examination of her pelvic lymph nodes under polarized light microscopy showed diffuse areas of birefringence compatible with talc, confirmed by scanning electron microscopy and X-ray spectroscopy.

CONCLUSION: This description of talc in pelvic lymph nodes of a woman with ovarian cancer and decades of exposure to talc may prompt new studies and offer new insights into the biologic basis for the consistent, but debated, association between talc use and ovarian cancer.
(Obstet Gynecol 2007;110:498–501)

An epidemiologic association between the use of cosmetic talc in genital hygiene and ovarian cancer was first described in 1982, and many subsequent studies found talc use to increase risk for ovarian cancer.[1] However, the causality of the relationship has been challenged for several reasons.[2] First, the association is a relatively weak one (ie, summary relative risk of approximately 1.3). Second, no clear increase in risk with duration of use has been found in most studies. Third, the ability of talc used in the genital area to enter the pelvic cavity has not been conclusively proven. At the time of pelvic surgery for ovarian cancer, pelvic lymph nodes are commonly sampled for staging purposes, but pathologic examination of the nodes is focused on the presence or absence of metastatic disease. More careful examination of pelvic lymph nodes from women with ovarian cancer may contribute to new perspectives in the debate regarding the role of talc in the causation of ovarian cancer, as illustrated by the following case.

CASE

A 68-year-old, married woman presented with abdominal swelling. A computed tomographic scan revealed a 13-cm pelvic mass, and her serum CA 125 level was more than 1,000. She was referred to the Gynecologic Oncology Service at the Brigham and Women's Hospital, where cytoreductive surgery was performed, including total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy, and pelvic lymph node sampling. A stage III papillary serous carcinoma with a minor clear cell component was found. Metastatic serous carcinoma was described in two of six right external iliac and obturator nodes. Postoperatively, the patient was referred for chemotherapy. She also consented to our interview about risk factors for ovarian cancer. This study is approved by the Dana Farber–Harvard Cancer Center Institutional Review Board and permits administration of general and dietary questionnaires, blood donation, and investigation of surgical specimen(s) after written informed consent. The patient's past history included three term deliveries followed by a tubal ligation. She had not smoked, used oral contraceptives, or used postmenopausal hormone therapy other than 6 months of progesterone therapy to regulate periods around the time of menopause, which occurred at age 50. There is a family history of colon cancer in a sister and maternal grandmother. At our interview, the patient stated she had used talc daily for 30 years as a body powder on the perineum and also applied it to underwear and sanitary napkins.

In searching for ideas to help clarify the association between talc use and ovarian cancer, we consulted with an expert on mesothelioma (J.G.), who pointed out that asbestos and other particulate material commonly migrates to lymph nodes.[3,4] We decided that a more systematic examination of pelvic lymph nodes from ovarian cancer cases might be in order, beginning with this case. In examining the patient's pelvic lymph nodes, no distinct particulates were seen under regular light microscopy, although a diffuse histocytic reaction was noted, even in a node without metastases (Fig. 1A). Under polarized light, diffuse

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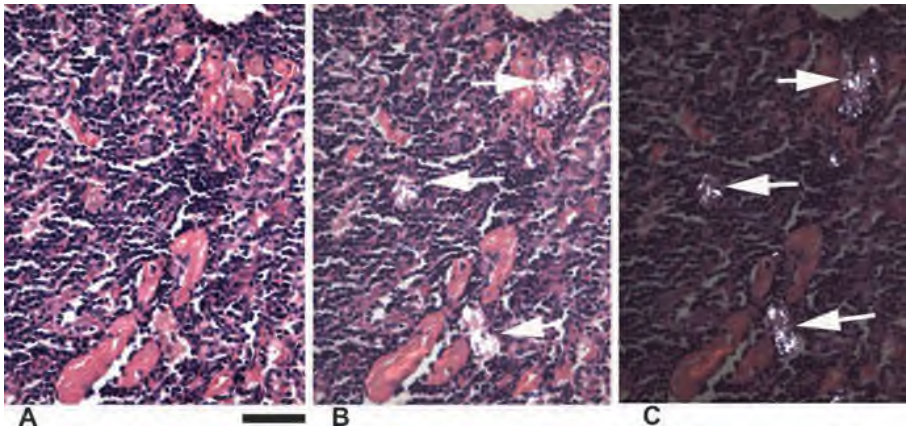

Fig. 1. Hematoxylin and eosin–stained section of a lymph node from the case showing morphologic findings with no polarization of the microscope light and with combinations of polarized and incident light at several different levels. **A.** Nodal morphology is illustrated and reveals no particulates seen without polarized light, but clusters of histiocytes are more prominent than usual. **B.** This panel shows the same field with polarized light plus additional light to view tissue context; birefringence is noted especially in areas of histiocyte clusters. *Arrows* are used to call attention to the birefringent particles. **C.** This shows the same field without added light, revealing the particulate nature of the birefringent material. *Arrows* highlight the particulate. Magnification bar shows 100 μm and applies to all three panels.

Cramer. Talc in Pelvic Lymph Nodes. Obstet Gynecol 2007.

birefringence was seen corresponding to the areas of histiocyte infiltration (Fig. 1B). Figure 1C shows the same field under polarization with no added light, revealing the particulate nature of the material, compatible with talc. Three of this patient's four nodes (not containing metastases) displayed polarizing material. Using methods described by Shelburne et al,[5] we next examined lymph nodes from this patient by combined scanning electron microscopy and energy dispersive X-ray spectroscopy. Scanning electron microscopy revealed plate-like particulates in the 5–10 μm range within the lymph node, in which energy dispersive X-ray spectroscopy showed a magnesium and silicate signature—compatible with talc (Fig. 2A,B). Dystrophic calcium deposits were also found within her nodes, probably a consequence of nodal aging. Of nodes from the next 12 patients examined, this case was strongest for birefringence; but these nodes have not yet been subjected to scanning electron microscopy or energy dispersive X-ray spectroscopy. Figure 3 illustrates a node negative for polarization (or histiocyte reaction) from a patient with ovarian cancer who had not used talc.

COMMENT

Talc is a hydrous magnesium silicate chemically similar to asbestos but structurally quite different. Asbestos has a fiber-like structure and talc a plate-like one. Because of this difference, it has been argued that the relationship between asbestos and mesothelioma should not be invoked to explain how talc might cause ovarian cancer. However, one feature of expo-

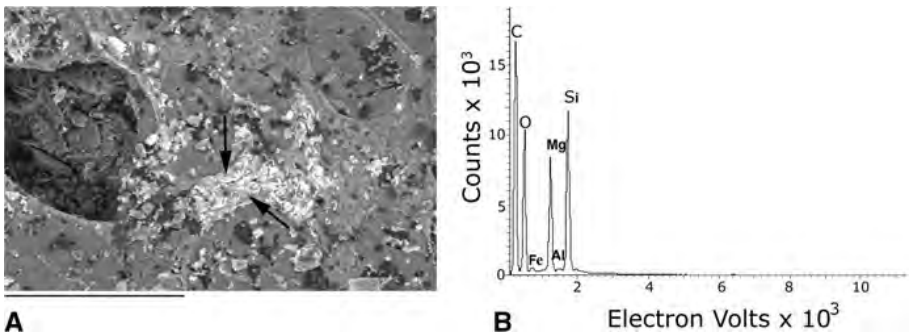

Fig. 2. Analytical microscopy. **A.** Scanning electron microscopy of a histologic section of the lymph node from the case shows a large collection of plate-like particulates in the 5–10 μm range *(arrows)* as well as scattered individual particulates. Magnification bar shows 100 μm. **B.** X-ray spectrum taken from the central bright area with particles reveals a Magnesium (Mg), Silicon (Si), and Oxygen (O) signature compatible with talc. A Carbon (C) signal is coming from the tissue or the underlying Carbon plancette or both.

Cramer. Talc in Pelvic Lymph Nodes. Obstet Gynecol 2007.

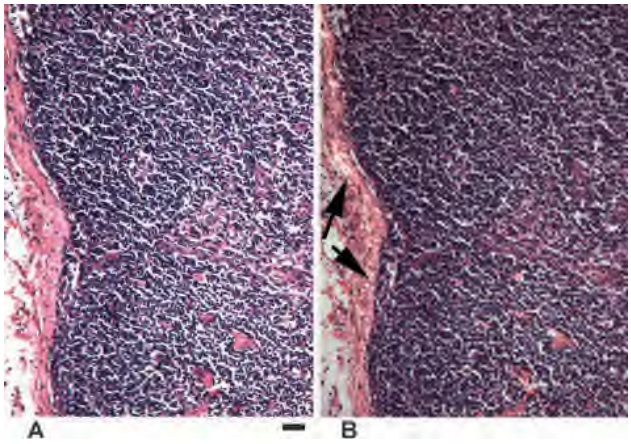

Fig. 3. Comparative node section illustrated from a woman reporting no talc use. **A.** Hematoxylin and eosin stained section showing fewer macrophages than seen in the node in Figure 1A. Magnification bar shows 100 μm. **B.** Polarized light examination of the same area of the node showing only some birefringence in the node capsule *(arrows)* compatible with collagen.

Cramer. Talc in Pelvic Lymph Nodes. Obstet Gynecol 2007.

sure that the minerals do have in common is nodal dissemination. Migration and entrapment in lymph nodes is observed in human asbestos exposure and correlates with the asbestos burden.[3] Talc has also been described in pulmonary lymph nodes of talc miners.[4] However, a MEDLINE search of (all language) publications between January 1950 and February 2007 using the search terms, "talc," "birefringence," "histiocytosis," "lymph nodes," and "ovarian neoplasms," revealed no reports of talc in lymph nodes of ovarian cancer patients.

In one of the few studies in women to evaluate the potential for talc to migrate into the pelvis, Heller et al studied normal ovaries from women having oophorectomy for benign disease.[6] The protocol involved a multistep process of tissue rehydration, blotting, drying, digestion, rehydration, centrifugation, and multiple washes. After this process, polarizing bodies were found in all ovarian specimens examined by light microscopy. By electron microscopy, tissues from 5 of 12 women who regularly used talc and 6 of 12 who had not were found to have particles consistent with talc. The investigators concluded that talc can be found in ovaries but that this does not correlate with genital talc use. Contamination that might have been introduced during extensive processing is a potential weakness of this study.

In this case report, we describe examination of pelvic lymph nodes from a woman with ovarian cancer who had been a long-term talc user. Particles compatible with talc were clearly visible under polarized light in regular hematoxylin and eosin–stained sections from her pelvic nodes, which were then shown by scanning electron microscopy and energy dispersive X-ray spectroscopy to be talc. Thus, as opposed to the aforementioned study, we focused on pelvic lymph nodes rather than ovaries; and talc was shown to be present in macrophages within the actual tissue, ruling out contamination during processing.

In reporting this case, we are not proposing that pelvic lymph nodes from women with ovarian cancer must now be subjected to electron microscopy. However, pathologists may wish to examine pelvic lymph nodes with evidence of histiocytic infiltrates by polarized light microscopy. Clear evidence of polarization may be reported so that clinicians can obtain information about potential talc exposure, if this information has not already been collected. Also we are not claiming that a causal relationship between ovarian cancer and talc use is proven for this case or in general. Because case reports cannot establish causality, we have begun a more extensive study of nodes with two purposes. First it is necessary to establish in a quantitative manner the likelihood of finding talc in lymph nodes of women with ovarian cancer and correlate this by whether they did or did not use talc. Second, studies of immune markers in nodes may help make the case for a causal connection.

What we do hope this case report accomplishes is to infuse a fresh perspective on the talc and ovarian cancer association. Previous biologic arguments linking talc and ovarian cancer have been based upon: similarities between talc and asbestos, the ability of talc to reach the ovaries through the open female tract, and induction of a mesothelioma-like cancer from the ovarian epithelium. Our new perspective would not depend upon structural similarities between talc and asbestos. The adverse effects of talc may relate to its ability to induce an inflammatory reaction, a well-established property of talc, independent of any similarity to asbestos.[7] Also, we don't believe that talc needs to reach the ovaries to affect ovarian cancer risk; rather, the harmful effects of talc may involve inflammatory reactions in the lower genital tract, including the upper vagina, cervix, and endometrium. These tissues express the surface glycoprotein human mucin 1, MUC1, whose function is to protect cells from environmental stressors. It is likely that chronic talc exposure is one factor that upregulates MUC1 expression. Human mucin 1 is related to CA 125 (MUC16), and like CA 125 is overexpressed in ovarian cancer. It is known that women with ovarian cancer who have anti-MUC1 antibodies survive longer, leading us to propose that

many risk factors for ovarian cancer may be explained by their ability to raise or lower MUC1 immunity.[8] Looking at predictors of anti-MUC1 antibodies, talc use was a factor that lowered anti-MUC1 antibodies. Thus, rather than a direct carcinogenic effect on ovarian epithelium, immune dysregulation involving MUC1 may be induced by chronic talc use that may lower protective immunity. Furthermore, sequestration of talc in nodes may affect antigen processing and be another important element in the postulated immune dysregulation.

In conclusion, this description of talc in pelvic lymph nodes of a long-term talc user with ovarian cancer may begin to reshape understanding about the relationship between talc and ovarian cancer and shed new light on whether talc used externally in the genital area is capable of migrating into the pelvis.

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Postpartum Sudden Death From Pulmonary Hypertension in the Setting of Portal Hypertension

Carlie S. Sigel, MD, Teresa C. Harper, MD, and Leigh B. Thorne, MD

BACKGROUND: Pulmonary arterial hypertension carries a high maternal mortality rate in the peripartum period. Pulmonary hypertension may arise as a complication of portal hypertension with poor patient survival.

CASE: A young primigravida with chronic autoimmune hepatitis and portal hypertension presented at 26 4/7 weeks of gestation with contractions and bleeding. Within 48 hours, an 892-g female fetus was delivered vaginally without complications. On postpartum day 2, the mother was found on the floor by her bed. Although initially responsive, within minutes she was unresponsive and resuscitation was unsuccessful. Postmortem examination showed cirrhosis and plexogenic pulmonary arteriopathy.

CONCLUSION: Increased awareness of pulmonary hypertension as a complication of portal hypertension and a high index of clinical suspicion are necessary to diagnose pregnant women with this condition and provide appropriate prenatal counseling and peripartum intervention.

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Pulmonary hypertension is an under-recognized complication of portal hypertension. We present an individual with known autoimmune hepatitis with cirrhosis and portal hypertension where underlying pulmonary hypertension was identified after her postpartum sudden death. Pulmonary hypertension may present in a subtle manner, but is important to appreciate in this high-risk obstetric patient population.

CASE

A young primigravida with a 10-year history of autoimmune hepatitis with chronic thrombocytopenia presented to the hospital at 26 4/7 weeks of gestation with contractions and bleeding. Before her pregnancy, she was a noncompliant transplantation candidate not using birth control. Prenatal care had been initiated at 6 weeks of

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Exhibit 52

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Correlative polarizing light and scanning electron microscopy for the assessment of talc in pelvic region lymph nodes

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Correlative polarizing light and scanning electron microscopy for the assessment of talc in pelvic region lymph nodes

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ABSTRACT

Perineal talc use is associated with ovarian carcinoma in many case-control studies. Such talc may migrate to pelvic organs and regional lymph nodes, with both clinical and legal significance. Our goal was to differentiate talc in pelvic lymph nodes due to exposure, versus contamination with talc in the laboratory. We studied 22 lymph nodes from ovarian tumor patients, some of which had documented talc exposure, to quantify talc using digestion of tissue taken from paraffin blocks and scanning electron microscopy/energy dispersive X-ray analysis (SEM/EDX). Talc particles correlated significantly with surface contamination assessments using polarized light microscopy. After adjusting for surface contamination, talc burdens in nodes correlated strongly with perineal talc use. In a separate group of lymph nodes, birefringent particles within the same plane of focus as the tissues in histological sections were highly correlated with talc particles within the tissue by *in situ* SEM/EDX ($r = 0.80$; $p < 0.0001$). We conclude that since talc can be a surface contaminant from tissue collection/preparation, digestion measurements may be influenced by contamination. Instead, because they preserve anatomic landmarks and permit identification of particles in cells and tissues, polarized light microscopy and *in situ* SEM/EDX are recommended to assess talc in lymph nodes.

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talc; scanning electron microscopy; carcinoma; birefringence

Introduction

In diseases related to foreign particulate exposure, accurate quantification of foreign material in tissue is important to document exposure and to correlate with disease occurrence or severity related to that tissue.[1] The issue is perhaps best appreciated for asbestos and pulmonary mesothelioma and fibrosis.[2] The most comprehensive quantification is obtained by digestion of a tissue sample, which uses much larger amounts of tissue that can be assessed in a histologic tissue section.[1] The procedure can be used to identify and quantify individual fibers by transmission electron microscopy (TEM) or scanning electron microscopy (SEM) and characterize them by energy dispersive x-ray analysis (EDX) to verify that their elemental signatures are compatible with a specific type of asbestos or other foreign material exposure[3] Application of TEM and/or SEM and EDX to tissue sections cut from paraffin blocks also provides quantification when the concentration of particles in tissue is sufficiently high.[4,5] This procedure may also show where the foreign material resides within a tissue section, such as exogenous particles localizing in macrophages within lymph nodes.[6] An estimate of foreign particulate exposure may also be obtained by studying histologic tissue sections under polarized light microscopy, which highlights birefringent material and its size and shape.[7,8] Besides the use of these methods in scientific studies to characterize exposures and disease, these techniques have also been used in medicolegal contexts related to claims of injury from various exposures, including asbestos.[1]

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Supplemental data for this article can be accessed here.

One exposure of great current medical, public health, and medicolegal importance is the association of ovarian cancers with the use of talc cosmetic products in the genital area. Data related to this association come from epidemiologic studies which identified a clear excess of women with ovarian malignancy who had used talc in their genital area prior to developing cancer, compared to control women.[9 13] The International Agency for Research on Cancer has declared the use of talc (not containing asbestos) in the genital area as possibly carcinogenic (Class 2B) (IARC monograph, 2010).[14] The most recent summary of the epidemiologic data in 2018 found that genital talc use may increase the risk for ovarian carcinoma by about 30%.[15] Although the origin of the hypothesis about talc and ovarian cancer came, in part, from description of talc in ovarian tissue,[16] demonstration that talc is present in the ovarian tissue or the genital tract from women with ovarian cancer has not been a component of the epidemiologic studies, and published data regarding talc in women's pelvic organs is very limited. A study by Heller et al.[17] was done with digestion techniques followed by TEM on ovaries from 24 women having hysterectomy/oophorectomy for reasons other than ovarian malignancy. This study found talc in approximately half the samples, with no obvious correlation with genital talc use history, thereby suggesting to the authors that talc exposure may be relatively ubiquitous across the population. A subset of authors from the present study have previously described a case report[6] in which a woman with serous carcinoma of the ovary, and a history of talc usage in her genital area, was demonstrated to have talc in three of four examined pelvic lymph nodes.

In the study reported here, we assessed talc in a sizable set of lymph nodes of the pelvic region, representing multiple patients. Thus, we expanded on the lymph node analysis in the previous case report[6] as well as the study of non-malignant ovaries by Heller et al.[17] and we examined nodes in 22 patients with various types of ovarian tumors. We included the additional step of an independent polarized light microscopy study on the histological sections for each case; this procedure assessed the quantity and location of birefringent particles in relationship to tissue landmarks. By digesting the lymph node samples, assessing the presence of talc by SEM/EDX, and comparing that data to the findings by light microscopy, we assessed tissue surface contamination as a factor explaining the high talc burden in some cases, as opposed to talc that migrated to the nodes from perineal exposure. We also endeavored, by studying a separate group of lymph node cases, to show that polarized light microscopy is a useful adjunct to *in situ* SEM/EDX, since both preserve anatomic landmarks and can serve as indicators of talc whose source is not due to contamination.

Materials and methods

Twenty-two women with ovarian tumors who had received their care in 2004 and 2005 at the Brigham and Women's Hospital (BWH), and who had participated in larger epidemiologic studies of ovarian cancer in Eastern Massachusetts and New Hampshire, were selected for the study. Women in this series were selected consecutively on the basis of meeting eligibility criteria and not on the basis of whether they had used talc. To be eligible, cases must have had lymph nodes removed from the pelvic region as part of their surgery. Cases were ineligible if the only nodes available contained metastatic disease or if there was only one unaffected node available. Though most of the cases were malignant ovarian neoplasms, two cases (one a borderline tumor and the second a granulosa cell tumor) were included because the study's objective was focused on the quantification of talc in tissue and understanding contamination vs. exposure related findings. Relevant clinical data were available both from the medical record and questionnaires completed by the women that included information on the use of talc in the genital area or as a body powder. The study was approved by the BWH Institutional Review Board and the informed consent signed by the women included permission to study material removed at the time of surgery. This group of women had both digestion studies and light microscopic studies of their lymph nodes. For our purposes, nodes of interest included inguinal, iliac, and paraaortic, and potentially any node of the pelvic region used for sampling and/or staging in ovarian surgical oncology. In some cases, the

designation "pelvic lymph node" with laterality, but without further anatomic specification, was provided with a sample.

Talc is readily visible under polarizing light microscopy, where it may be found as both plates and fibrous forms, and where the particles or fibers are brightly birefringent and often in the size range 1–10 μm. Identification of talc by electron microscopy and energy-dispersive X-ray analysis (EDX), includes the plate-like particulate or fiber-like structure and a spectrum showing magnesium and silicon peaks within 5% of the theoretical atomic ratio of 0.75 and atomic weight percent ratio of 0.649.

For each patient case, we ascertained that an acceptable representative hematoxylin-eosin (H&E)-stained slide was available for the block prior to subsequent steps. Tissue was totally cut from the paraffin block with a cleaned scalpel, heat deparaffinized, and then multiple extractions were done with xylene to remove all residual paraffin. The tissue was weighed, then added to glass centrifuge tubes, and sodium hypochlorite solution was added for digestion over a 24–48 hr period. When digestion was complete, samples were centrifuged and the sediment resuspended in filtered distilled water and vortexed until no sediment was visible. The tubes were centrifuged again and the supernatant aspirated. Sediments were resuspended in 25% ethanol, mixed by vortexing and filtered through a 13 mm, 0.2 μm Millipore filter. Tubes were washed twice with 25% ethanol and filtered. Filters were dried in a desiccator and were mounted on a carbon planchette.

Samples were analyzed in a scanning electron microscope (Leo 1460VP) equipped with an EDX spectrometer (Oxford instruments with Inca software) or an Hitachi SU6600 field emission scanning electron microscope with Oxford EDX (Xmax 50SDD EDX detector) and Oxford instrumentation software (Aztec 3.3). At 2000x magnification, 200 particles or 100 random fields were analyzed for each case, whichever came first. Using various parameters, including the number of talc particles identified by their chemical composition, the area of each microscopic field times the number of fields examined, and the overall filter area, an estimate for the total number of talc particles in the specimen was calculated.

Because fat, fibrous tissue, and lymph node contributed to the weight of the material used for digestion and because there were differences in birefringent particle distribution patterns of the tissue surface, fat and fibrous tissue, and lymph node, a more accurate approach was needed by which we could estimate the contributions of the separate locations. Tissues on all slides were digitized. Using NIH Image J analysis software (an open source image processing program, www.imagej.com), the total areas (cm^2) of the tissue on the slides for each case were calculated, as well as the respective components of lymph node and fibroadipose (soft) tissue, with the sum of these areas adding up to the total tissue area. These figures were then multiplied by 0.25 cm (a typical thickness for tissue in paraffin cassettes from which the digested tissues were derived) to obtain total specimen volumes for the total tissue, and for the lymph node and soft tissue components. The total number of talc particles identified in the digestate by SEM was then divided by the total tissue volume to obtain the number of talc particles per unit volume (cm^3).

H&E slides of intact lymph node tissue corresponding to each digested paraffin sample were analyzed with an Olympus BH-2 light microscope equipped with polarizing filter capabilities (analyzer and rotating polarizer with specimen slide in between). Each slide was scanned systematically and completely at 200x magnification under polarized light. Slides typically contained one to several lymph node profiles with adherent fibroadipose tissue. Birefringent particles visually consistent with talc (typically 1–10 μm with birefringence) were counted that were located within the lymph node parenchyma and sinuses, and a separate count was made of particles in fibroadipose (soft) tissue, i.e. not within the lymph nodes proper. The counts of these two components were added to get the total count. Particles within fibroadipose tissue were counted only if they were at least one 400x (high-power) field away from the surface, so that obvious surface contamination was not included in the counts. The birefringent particles present within lymph nodes were taken to indicate clinically significant talc that migrated there through the lymphatic system. Birefringent particles on the physical surface of the tissues were not counted for these analyses but instead assessed as described below.

Using the aforementioned image analysis data which provided the areas (cm^2) for the total tissue on the slide as well as the lymph node and soft tissue components, for each slide, the respective tissue volumes were calculated by multiplying the areas by 4 μm (4×10^{-4} cm), a standard tissue section thickness on glass slides. The number of birefringent particles per unit volume were then calculated (through simple division) for each tissue component and for the overall tissue. This meant that the volume correction factor between tissue blocks and tissue slides was approximately 625 (0.25 cm thickness of tissue in blocks vs. 4 μm thickness of slides).

Additionally, for each of the 22 cases, a semi-quantitative visual estimate of surface contamination was made. This was done by observing the quantity and pattern of all polarizable material (typically birefringent particles of 1–10 μm, plus larger material such as paper, organic fibers, and other debris) that were present along the specimen edge and/or within one 400x (high power microscopic field) width from it. The objective here was to measure the degree to which the specimen surfaces might have been contaminated by physical manipulation during the acquisition and handling steps of the specimen in the Pathology department. Our estimate scores ranged from 0 to 3 and the criteria for the scoring was as follows (see Figure 1): 0, no polarizable material along surface; 1, occasional foreign particulates, rarely forming small clusters; 2, moderate numbers of surface particulates, forming occasional clusters or surface patches more numerous than in score 1; 3, frequent patches of particulates along with confluent stretches of contamination along the surface. Typically, such contamination was seen along the fibroadipose tissue surface with the nodal tissue interior to that. The contamination consisted typically of a mix of larger debris consistent with paper, along with smaller birefringent particulates similar to those seen and described in tissue sections (Figure 1). All contamination scores were done by a pathologist (JJG) in a blinded fashion (SEM and clinical data were unavailable at the time of scoring). A randomly chosen subset of the same cases was independently scored by a second pathologist (SM), also in a blinded fashion, to confirm successfully that the review standards agreed, and thus the scoring standards were being applied consistently.

Subsequent statistical analysis for the 22 cases was handled as follows: Talc counts were log transformed to create normal distributions. Spearman correlations were calculated to assess the relationship between potential contamination on the talc counts and each continuous variable, and partial correlations were used to examine the relationships between talc counts, adjusted for contamination. Linear regression was used to calculate crude and contamination-adjusted talc/total volume geometric means and 95% confidence intervals.

Also, as part of this report, we studied a second group of 19 lymph node specimens from 10 ovarian carcinoma cases. The 10 cases were consults of authors JJG and WW, which were de-identified, i.e. reported here without any patient identifiers, including the 18 recognized HIPAA identifiers.[18] All 19 tissue specimens had histologic slides and corresponding paraffin blocks available. In this component of the study, we assessed the relationship of the numbers of birefringent particles in the lymph node parenchyma in histological sections, and talc particles found by *in situ* SEM/EDX at deeper levels in the tissue blocks corresponding to those sections. Digestion was not performed on these cases; nor was information available on their talc exposure. Birefringent particles in the lymph nodes were exhaustively quantified by light microscopy as previously described (particles counted in respective lymph node and soft tissue components, added to a total count for each slide). The histologic slides typically contained from one to several lymph node profiles, each with adherent fibroadipose tissue. Counting was done without regard to the number of profiles; i.e. an aggregate count was obtained across all lymph node tissue on a slide.

The tissue blocks were handled with a procedure for *in situ* SEM/EDX distinct from the tissue digestion and filter analysis by SEM described in the previous component of the study. This *in situ* procedure was first described by Thakral and Abraham[4] for assessment of particulate materials in paraffin-embedded tissue. In the study reported here, the blocks were handled with particle-free gloves on pre-cleaned surfaces and sectioned removing ~30 micrometers of tissue

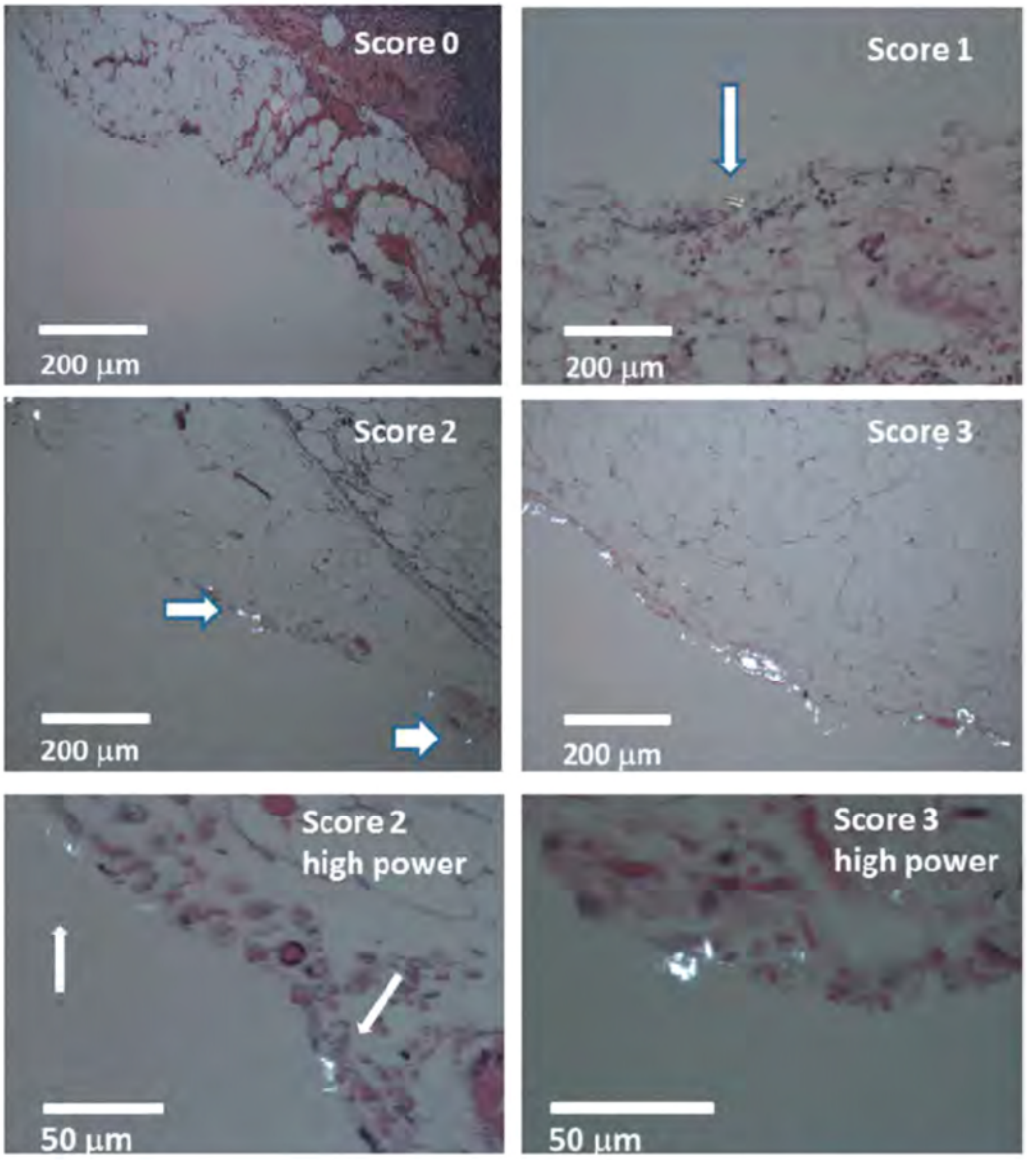

Figure 1. Tissue surface contamination score semi-quantitative grading. As shown especially in the two high-power images at bottom, the contamination material consisted typically of larger debris consistent with paper, along with smaller birefringent particulates. Surface contamination was typically found along the fibroadipose tissue surface, with lymph node tissue located underneath. Grading scheme is as follows: **Score 0**: no polarizable material along surface. **Score 1**: occasional birefringent particulates (arrows), rarely forming small clusters. **Score 2**: moderate numbers of surface birefringent particulates (arrows), forming occasional clusters or surface patches more numerous than in score 1. **Score 3**: frequent patches of particulates along with confluent stretches of contamination along the surface. (All images under polarizing light microscopy, H&E staining, all 100x except 400x [original magnification] in the bottom two images which respectively show score 2 and 3).

and paraffin using a rotary microtome with a new, clean stainless-steel blade. This sectioning was intended to remove any surface contamination from previous storage and handling. After the fresh surface was exposed, the block surfaces were washed in distilled, deionized water for 30 seconds to remove soluble surface materials such as sodium chloride and sodium phosphates used in processing for histology. The blocks were mounted for SEM examination and always kept in closed containers to limit any lab contamination. These tissue surfaces were studied with a Hitachi SU6600 field emission SEM with an Oxford EDX with Aztec version 2.0 to 3.3 software, and EDX detector model X-Max 50 SDD. The backscatter mode of the microscope highlighted mineral particles within the tissues. Areas of the tissue at the sectioned block surface were

examined at relatively low magnification 200-500x, and when particles were seen, they were then examined at higher magnification for morphological characteristics and to carry out spectral analysis on each particle found. Electron beam penetration depth under the conditions used was estimated to be 2.5 μm, with an analysis range of 0.5–2.5 μm. Of note, under *in situ* SEM the interior tissue and exterior tissue surfaces were readily distinguishable; this distinction was important for our study. In particular, as subsequent discussion will show, it was important to avoid analyzing surface particulates and instead analyze those inside the tissue. Having a scanned photocopy of the light microscopic slide and the block surface available for reference when performing SEM/EDX helped in navigating the anatomic landmarks, including surface vs. tissue interior location. We subsequently carried out an auxiliary part of this study, in which surface contamination of tissue slides was assessed using two of the cases that had this finding. The surface particles were assessed by *in situ* SEM/EDX to determine the identity (i.e. chemical composition) of the surface contamination.

For this second part of the study, linear regression analyses, with the generation of a coefficient of determination (r) goodness-of-fit value, were done between three statistical pairings: total birefringent particles by light microscopy vs. *in situ* SEM/EDX talc counts, lymph node birefringent particles vs. *in situ* SEM/EDX talc count, and fibroadipose tissue birefringent particles vs. *in situ* SEM/EDX talc counts. Our hypothesis was that the first two pairings would be correlated but the last one would not. The inclusion of multiple specimens from some of the patients meant that the 19 data points (specimens) were not truly independent of each other from the perspective of the population. However, from a statistical point of view, this was justified because, in this phase of our study, the purpose was an evaluation of methods and data related to the samples themselves, and not the population from which the samples were drawn.

Results

Digestion study

Table 1 shows characteristics of the 22 subjects enrolled in the BWH node digestion study, arrayed (least to greatest) by the amount of talc (by digestion) per cm^3 tissue volume. Fourteen (64%) of the women had invasive serous ovarian carcinoma of the ovary, which in one case was mixed with endometrioid carcinoma. Nineteen of the 22 nodes (86%) were external iliac, with 11/19 (58%) from the right side. The age range of the women was 38–73 with a median of 56; 10 (45%) had used talc in their genital area and 16 (73%) had used it as a body powder. There was considerable variation in total talc counts seen after digestion of the nodes. There was also considerable variation in birefringent particle counts in the nodal components, as well as corresponding counts per cm^3 tissue volume (see column totals where pertinent). The number and proportion of nodes with 0, 1, 2, and 3 surface contamination scores were: 4(18%), 7(32%), 7(32%), and 4 (18%).

Of note, cases 4, 9, and 13 had no clinical exposure history, and yet all had high contamination scores (either 2 or 3) and corresponding moderate to high talc counts per cm^3 tissue volume, thus highlighting a role for contamination in their digestion results. In contrast, cases 10 and 18 had clinical **exposure, but** zero contamination scores (i.e. no visible surface contamination); they also had significant talc counts per cm^3 tissue volume, indicating that in the absence of surface contamination, clinical exposure yields significant talc counts using digestion. Case 18 can also be contrasted with cases 19–22, which had the four highest talc counts per cm^3 tissue volume (Table 1), and all of which had high levels of surface contamination.

Table 2 shows Pearson and partial correlations among the various quantitative measurements related to talc and birefringent particles. The degree of surface contamination (0–3 score) as it correlates with other measures of talc and birefringent particles within the node is shown in the right-most column. The surface contamination score was significantly correlated with: the total talc particle count by digestion (r 0.43, p 0.05); with birefringent particle counts by light microscopy in the soft tissue (fibroadipose) component (r 0.53, p 0.01); with total talc per cm^3 tissue volume by SEM/EDX (r 0.57, p 0.006); and with birefringent particle counts in fibroadipose tissue per cm^3 fibroadipose volume (r 0.51, p 0.01). The remainder of correlations and p values in Table 2 represent those for partial correlations

Table 1. C inica data and ta c digestion and ight microscopic data among the first patient group (BWH cases).

Case number	Tumor histo ogy	Node*	Component vo ume (cm^3)			Age	Ta c use		Tota ta c †	Ta c/cm^3 of tissue vo ume	Tota birefringence counts††			Birefringence per component vo ume (partic es/ cm^3)			Surface contamination
			Tota	Node	Fat		Genita	Body			Tota	Node	Fat	Tota	Node	Fat	
1	Endometrioid	RE	0.341	0.195 (57%)	0.146 (43%)	60	No	Yes	844	2,475	3750	1250	2500	11,000	6,375	17,250	1
2	Serous invasive	LP	0.334	0.171 (51%)	0.164 (49%)	53	No	Yes	1608	4,800	1250	625	625	3,737	3,661	3,812	1
3	Serous invasive	LE	0.308	0.119 (39%)	0.188 (61%)	69	No	Yes	2065	6,705	10625	6250	4375	34,552	52,301	23,271	0
4	Serous invasive	LE	0.407	0.252 (62%)	0.155 (38%)	38	No	No	4290	10,540	4375	1250	3125	11,187	4,960	20,187	2
5	C ear ce	RE	0.332	0.189 (57%)	0.143 (43%)	54	No	Yes	3965	11,942	15000	12500	2500	45,146	66,286	17,406	0
6	Serous invasive	RE	0.232	0.169 (73%)	0.063 (27%)	50	Yes	No	3378	14,500	1250	625	625	5,387	3,687	9,937	1
7	Endometrioid	RE	0.557	0.392 (70%)	0.165 (30%)	46	No	No	8920	16,000	4375	1250	3125	7,912	3,187	18,937	1
8	Serous invasive	LE	0.107	0.039 (36%)	0.069 (64%)	49	Yes	Yes	2533	23,562	1250	0	1250	11,687	0	18,375	1
9	Endometrioid	RE	0.533	0.089 (17%)	0.444 (83%)	57	No	No	19,094	35,823	15000	3125	11875	28,103	35,014	26,715	2
10	Granu osa ce	RE	0.237	0.206 (87%)	0.030 (13%)	49	Yes	Yes	20,267	85,600	4,375	3,125	1,250	18,500	15,125	41,375	0
11	Serous invasive	RE	0.107	0.092 (86%)	0.015 (14%)	51	No	No	10,390	97,100	5,000	625	4,375	46,750	6,812	291,687	2
12	Serous invasive	RP	0.026	0.021 (79%)	0.006 (21%)	51	Yes	Yes	2,834	107,300	10,625	5,625	5,000	402,437	269,125	908,750	2
13	Serous invasive	LE	0.147	0.022 (15%)	0.125 (85%)	68	No	No	16,057	115,030	16,875	1,250	15,625	114,812	56,562	125,125	3
14	Serous invasive	RE	0.219	0.145 (66%)	0.074 (34%)	73	Yes	Yes	30,330	138,500	8,125	1,875	6,250	37,062	12,937	84,437	2
15	Endometrioid	RE	0.506	0.083 (16%)	0.423 (84%)	58	Yes	Yes	73,267	144,800	26,875	12,500	14,375	53,125	151,500	33,937	2
16	Serous border ine	RE	0.147	0.055 (37%)	0.092 (63%)	60	No	Yes	21,409	145,600	11,875	2,500	9,375	80,812	45,437	101,875	1
17	Serous invasive	LE	0.174	0.123 (71%)	0.051 (29%)	62	Yes	Yes	33,778	194,100	30,625	28,125	2,500	176,000	228,687	49,437	1
18	Serous invasive	LE	0.323	0.203 (63%)	0.121 (37%)	53	Yes	Yes	67,557	208,200	>125,000	>125,000	625	>387,000	>616,365	3,000	0
19	Serous invasive	LE	0.052	0.017 (33%)	0.035 (67%)	69	No	Yes	12,661	242,100	11,250	1,250	10,000	215,000	71,875	285,625	3
20	Serous invasive	LE	0.286	0.185 (65%)	0.101 (35%)	66	Yes	Yes	92,891	325,200	4,375	3,125	1,250	15,312	16,875	12,437	2
21	Endometrioid	RE	0.056	0.039 (70%)	0.017 (30%)	51	No	Yes	85,041	1,518,589	13,750	1,250	12,500	246,875	32,051	735,294	3
22	Serous/ endometrioid	RPA	0.424	0.284 (67%)	0.139 (33%)	69	Yes	Yes	797,171	1,881,500	>62,500	>62,500	1,250	>147,500	>220,062	9,000	3
Median			0.262	0.134	0.111	56			14,359	102,200	10,625	2,188	3,125	41,104	33,533	24,993	

*Location of Node: LE = Left externa i iac; RE = Right externa i iac; RPA = Right paraaortic; LP = Left pe vic; RP = Right pe vic
†Tota number of ta c partic es by digestion (ca cu ated)
††Tota birefringence counts = partic es in fie d x 625 (see Materia s and Methods)
Node refers to ymph node parenchyma areas as measured by mage J software and studied by ight microscopy (see Materia s and Methods).
Fat refers to fibroadipose soft tissue areas as measured by mage J software and studied by ight microscopy

Table 2. Correlations between surface contamination, talc, and age (r and p values).

Variable*	Surface contamination r (p)	Total talc by digestion r (p)	Total birefringent particle counts			Talc/total volume	Birefringent particle counts per cm³ volume		
			Total r (p)	Node r (p)	Fat r (p)	r (p)	Total r (p)	Node r (p)	Fat r (p)
Total talc by digestion	0.43 (0.05)								
Total birefringent particle counts	0.15 (0.51)	0.67 (0.001)							
Total birefringent particle counts, node	−0.07 (0.77)	0.59 (0.005)	0.81 (<.0001)						
Total birefringent particle counts, fat	0.53 (0.01)	−0.13 (0.58)	0.25 (0.26)	0.07 (0.76)					
Talc/cm³ volume	0.57 (0.006)	0.87 (<.0001)	0.63 (0.002)	0.47 (0.03)	−0.06 (0.78)				
Birefringent particles per cm³ total volume	0.33 (0.13)	0.42 (0.06)	0.82 (<.0001)	0.56 (0.008)	0.3 (0.19)	0.68 (0.0007)			
Birefringence per cm³ node volume	0.07 (0.77)	0.51 (0.02)	0.90 (<.0001)	0.88 (<.0001)	0.18 (0.45)	0.64 (0.003)	0.87 (<.0001)		
Birefringence per cm³ fat volume	0.51 (0.01)	−0.24 (0.30)	0.003 (0.99)	−0.1 (0.68)	0.61 (0.003)	0.16 (0.48)	0.45 (0.04)	0.13 (0.58)	
Age	0.28 (0.20)	0.26 (0.26)	0.35 (0.12)	0.32 (0.15)	0.16 (0.49)	0.22 (0.33)	0.26 (0.25)	0.36 (0.12)	−0.07 (0.75)

Node = ymph node tissue
Fat = fibroadipose tissue

adjusted for the level of surface contamination. Not unexpectedly, total counts always strongly correlated with counts per cm^3 of relevant tissues: e.g. total talc with total talc per cm^3 tissue volume (r 0.87, p 0.001); total birefringent particle counts in lymph node tissue with birefringent counts per cm^3 lymph node tissue (r 0.88, p 0.0001); and birefringent particle counts in fibroadipose tissue with birefringence counts per cm^3 fibroadipose volume (r 0.61, p 0.003). Talc counts per cm^3 tissue volume correlated with: birefringent particles per cm^3 tissue volume (r 0.68, p 0.007), and lymph node birefringent particles per cm^3 lymph node tissue (r 0.64, p 0.003), but not with fibroadipose birefringent particles per cm^3 fibroadipose tissue. Total birefringent particles per cm^3 tissue volume correlated best with lymph node birefringent particles per cm^3 lymph node tissue (r 0.89, p 0.001). Birefringent particle counts per cm^3 lymph node tissue were not correlated with fibroadipose birefringent particle counts per cm^3 fibroadipose volume. Age was not significantly correlated with any measure of nodal contamination.

Figure 2 and Table 3 illustrates the potential effect of surface contamination on the interpretation of the relationship between total talc (by digestion) per cm^3 tissue volume. Figure 2 illustrates that for any level of surface contamination, those who used talc in the genital area had a higher amount of talc than those who had not used talc genitally. Table 3 quantifies

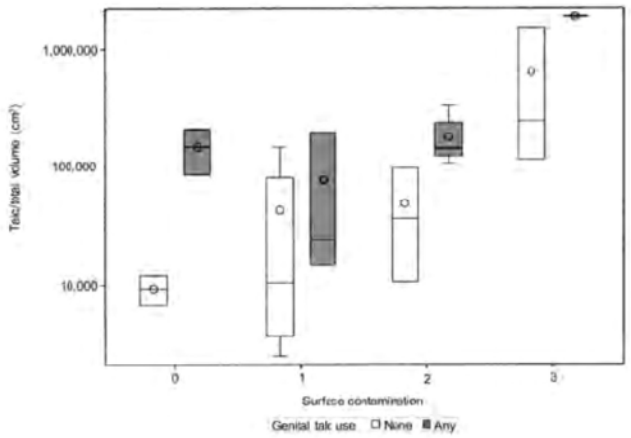

Figure 2. Talc/total volume for genital talc users and non-users by surface contamination This figure shows surface contamination scores (x axis) plotted against talc per tissue volume (y-axis, logarithmic scale), showing that for any level of surface contamination, those who used talc in the genital area had a higher amount of talc than those who had not used talc genitally.

Table 3. Geometric mean talc/total volume by genital talc use.

Talc/total volume	No genital talc use (n = 12) Geometric mean (95% CI)	Any genital talc use (n = 10) Geometric mean (95% CI)	p-value
Crude	35,049 (13,637, 90,079)	131,584 (46,787, 370,070)	0.08
Adjusted for surface contamination	29,926 (15,546, 57,605)	159,056 (77,491, 326,475)	0.004

this effect more precisely and indicates that, overall, the genital talc user had higher talc counts per volume of tissue than those who had not used talc, but the association was of borderline significance. After adjustment for level of surface contamination, the association became significant (p 0.004) with the level of talc in nodal tissue at least five times higher in those who used talc genitally compared to those who had not.

Figure 3 shows correlative polarizing light microscopy, SEM, and EDX from case 18 in the digestate study (Table 1). Going clockwise from upper left, panel **A** shows polarized light microscopy (H&E, 200x), showing numerous birefringent particles (general size range 1 to 5 µm) within the macrophages of a left external iliac lymph node. This case was near the upper end of the range of particle abundances we observed. Panel **B** shows examples of two particles (labeled 1103 and 1104), identified by SEM on the digestate filter, each <5 µm diameter. Panel **C** shows the spectrum for particle 1103, with an Mg-Si atomic weight ratio of 0.6495, characteristic of talc. The other particle in **B**, 1104, had an Mg-Si ratio within 5% of the theoretical talc value (0.649).

In situ *SEM study*

Table 4 shows data for the second part of the study (19 lymph node specimens from 10 patients). The leftmost two columns (case number and block letter) are

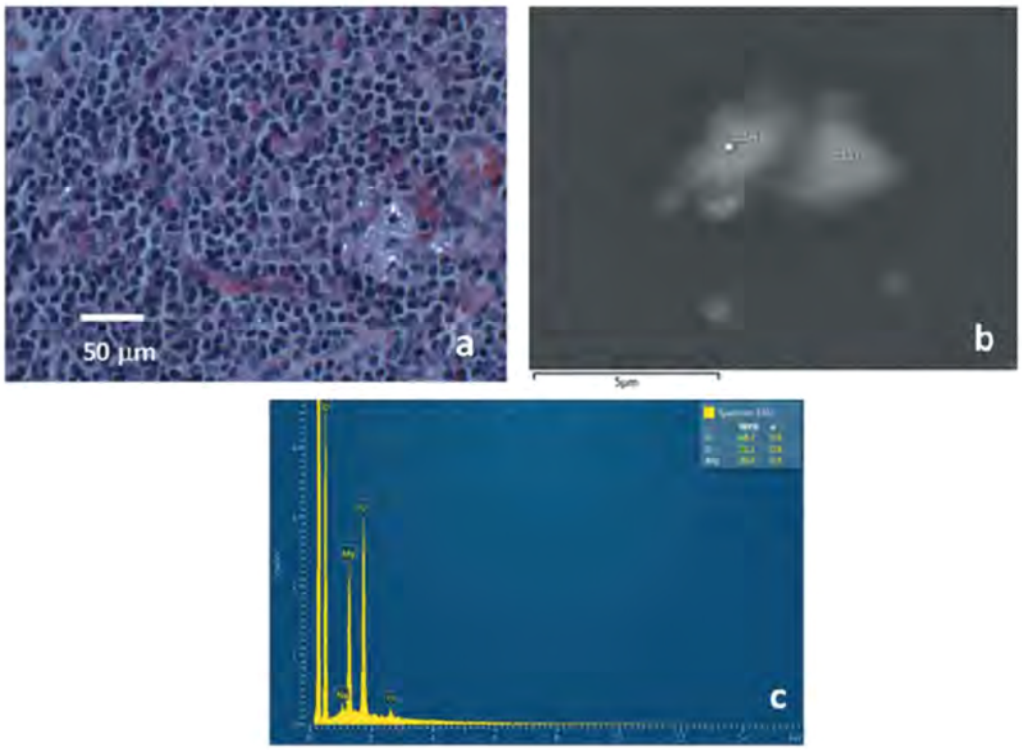

Figure 3. Correlative polarizing light microscopy, SEM, and EDX from case 18 in the digestate study (Table 1). Clockwise from upper left: **a**, Polarizing light microscopy, H&E, 200x, showing numerous birefringent particles (general size range 1 to 5 µm) within the macrophages of a left eternal iliac lymph node. **b**, Two particles (labeled 1103 and 1104), identified by SEM on the digestate filter, each <5 µm diameter. **c**, Spectrum for particle 1103, The Mg-Si atomic weight ratio is 0.6495, characteristic of talc. The other particle in **b**, 1104, had an Mg-Si atomic weight ratio within 5% of the theoretical talc value (0.649).

Table 4. Correlation between light microscopic birefringent particulates and in situ SEM analysis for talc particles.

Case number	Slide letter	Birefringent particles in lymph node tissue (total per slide)	Birefringent particles in surrounding fibroadipose tissue (total per slide)	Total birefringent particles in slide (columns C + D)	Number of talc particles in the block by in situ SEM
1	A	3	5	8	0
	B	55	7	62	5
2	A	5	2	7	9
3	A	2	0	2	0
	B	0	0	0	0
4	A	19	9	28	31
	B	3	1	4	5
5	A	>500	3	>500	65
6	A	6	4	10	0
	B	8	3	11	0
	C	16	4	20	0
7	A	7	3	10	1
	B	1	0	1	0
8	A	>100	3	>100	18
	B	>200	2	>200	43
	C	>100	5	>100	35
	D	>100	7	>100	24
9	A	8	6	14	1
10	A	15	>50	>50	12

In this part of the study, 19 pelvic lymph node slides on 10 ovarian carcinoma patients (with each patient having from one to four node specimens), showed the relationship of the numbers of birefringent particles (by light microscopy) within histological sections (separately categorized in lymph node and fibroadipose tissue components), and talc particles found by SEM/EDX at deeper levels in the tissue blocks corresponding to those sections (right-hand column). In case 9C, the vast majority of the birefringent particles were localized in only one of several lymph nodes visible in the slide. Note that cases with very numerous particle counts by light microscopy are designated simply as greater than a certain threshold.

fully de-identified and serve for identification purposes within the table only. The table shows the relationship of the numbers of birefringent particles by light microscopy within histological sections (separately categorized in lymph node and fibroadipose tissue components), and talc particles found by SEM/EDX on the block surface (following the preparation procedure) corresponding to those sections (right-most column). Consistent with our hypotheses, strong correlations using Spearman correlations were indeed evident between a) lymph node counts by light microscopy and the SEM total talc count (r 0.80, $p < 0.0001$); and b) total particle counts by light microscopy and the SEM total talc count (r 0.79, $p < 0.0001$). Fibroadipose tissue counts by light microscopy did not correlate with SEM total talc counts (r 0.32, p not significant). In controlling for correlated observations from the same patient, Spearman correlations using one record per case were done for the six patients where more than one lymph node specimen was included in the study (among these patients, the specimen with the highest SEM talc count was the one selected). With this adjustment, strong correlations were still observed using Spearman correlations as evident between a) lymph node counts by light microscopy and the SEM total talc count (r 0.69, $p < 0.03$); and b) total particle counts by light microscopy and the SEM total talc count (r 0.74, $p < 0.01$), Fibroadipose tissue counts by light microscopy did not correlate with SEM total talc counts (r 0.16, p not significant).

Figure 4 shows correlative polarizing light microscopy, *in situ* SEM, and EDX on case 9C from Table 4. Going clockwise from lower left, panel **A** shows numerous birefringent particles under polarized light microscopy (H&E, 400x) within the macrophages of a left external iliac lymph node. Panel **B** shows low-power backscattered electron imaging under SEM with several positive particles. Panel **C** shows an enlarged (cropped) view of the lower right-hand part of panel B. Three particles are labeled – 44, 45, and 46. Panel **D** shows the spectrum for particle 45, which showed an Mg-Si ratio of 0.643. Particle 44 was also within the 5% of the theoretical value of 0.649 and so was considered talc as well. Particle 46 had an Mg-Si ratio of 0.610, which falls just outside the 0.649 ± 5% range for talc, and so it was considered a nonspecific magnesium silicate.

A review of the non-talc particles found by *in situ* SEM in the 10 patients in Table 4 showed an aggregated total of 310, which based on their chemical composition would be regarded as likely birefringent. Of these, the most common were magnesium silicates outside the 5% theoretical range of the Mg-Si atomic weight spectral ratio for talc (113 total particles or 36%), aluminum silicates with or without magnesium (91 total particles or 29%), and calcium without phosphate (41, or 13%), with others accounting for the remaining 22%. Non-fibrous, non-talc silicates are known to have a longer dissolution time than talc in physiologic conditions; the dissolution time for talc is approximately 8 years for a 1 µm particle.[19] Thus, the component of non-talc silicates in pelvic tissues could proportionally rise over sufficient

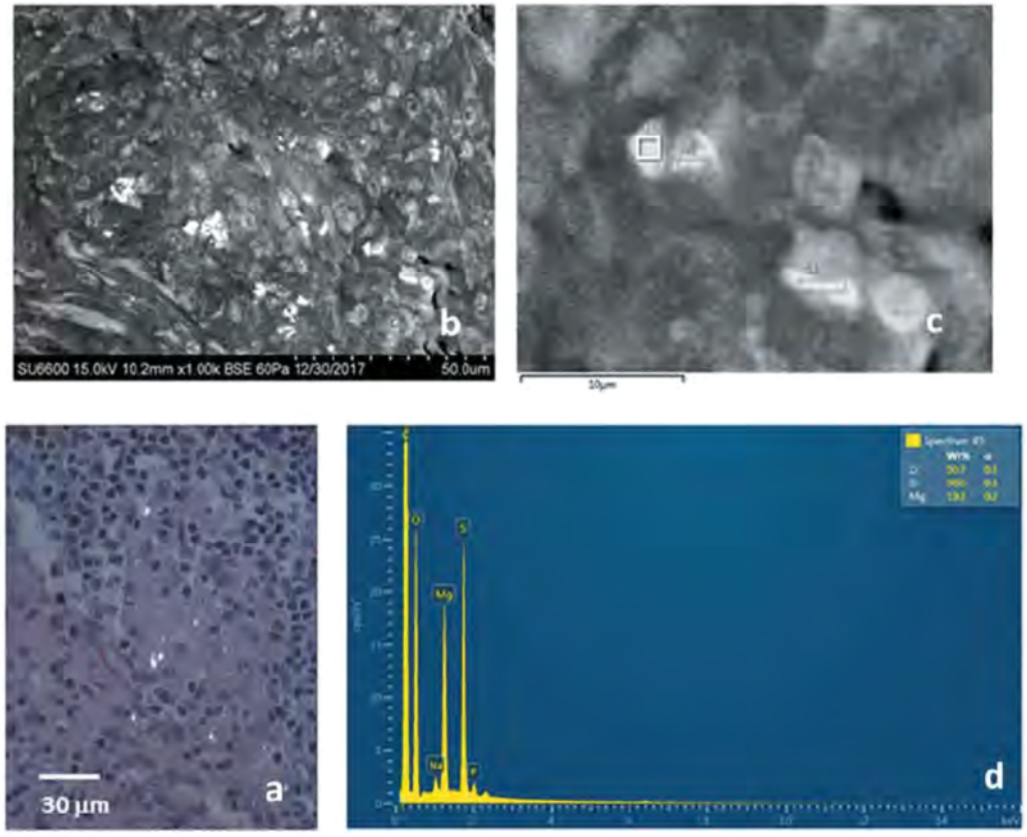

Figure 4. Correlative polarizing light microscopy, in situ SEM, and EDX on case 8C from Table 4. Clockwise from lower left: **a**, Numerous birefringent particles under polarized light microscopy (H&E, 400x) within the macrophages of a left external iliac lymph node. **b**, Low-power backscattered electron imaging under SEM with several positive particles. **c**, Enlarged (cropped) lower right-hand portion of **b**. Three particles are labeled – 44, 45, and 46. **d**, Spectrum for particle 45, which showed an Mg-Si atomic weight ratio of 0.643. Particle 44 was also within the 5% of the theoretical value of 0.649 and so can be considered talc as well. Particle 46 had an Mg-Si atomic weight ratio of 0.610, which falls just outside the 5% range for talc and so can be considered a nonspecific magnesium silicate.

elapsed time (years), even if the original exposure to talc was heavy.

To provide final evidence for our hypothesis that talc is an important part of specimen surface contamination, two authors (SM and JG) re-reviewed the 19 slides from the second part of the study (*in situ* SEM). The goal was to find cases in this group with surface contamination. We did not find any with a score of 3, but two cases (1B and 7A from Table 4) were chosen that, respectively, had contamination scores of 2 and 1 (with 100% agreement by pathologists SM and JG), and substantial amounts of evaluable surface area. On polarizing light microscopy, these cases showed a mixture of larger paper debris fragments and smaller (1–10 µm) birefringent particulates along the surface similar to those previously seen for many Table 1 cases., Respectively, for 1B and 7A, 13 and 5 small birefringent particulates were found by thorough examination of their surfaces in addition to larger paper debris. SEM of the tissue surface for block 1B (35 mm^2 analysis area) showed a total of 5 talc particles, and for 7A showed 1 talc particle (50 mm^2 analysis area). Given the 2.5 µm effective section thickness (electron beam analysis depth) and these relatively small surface areas, these SEM talc particle counts are significant, and are consistent with the light microscopic review. Thus, this portion of the study directly showed that surface contamination particles were talc, whereas previously, this had only been strongly implied by the results in Table 1. (See supplementary figure S1). In addition to the talc particles, 44 other exogenous particles were found across tissue surfaces of these two cases by SEM/EDX: 27 external mineral (mainly Si in combination with Mg and/or Al), 6 non-talc Mg-Si minerals, and 11 external metal.

Discussion

The accurate identification of talc in pelvic tissues is important because it documents exposure by demonstrating the presence of talc in these tissues and provides evidence in support of the role of talc in the epidemiological association with ovarian cancer in case-control studies.[9 13,15] The overall relative risk across the various positive studies is around 1.3, and where tumor histology data have been available for review, several common subtypes (serous carcinoma, endometrioid carcinoma, and serous borderline tumors) are most frequently involved in the association.[11,13]

Talc, when applied to the perineum, is believed to migrate to the upper genital tract, passing through the open tract to the fallopian tubes and eventually reaching the ovaries.[11,16] Talc may also gain access to the lymphatic system as a means of reaching pelvic organs and lymph nodes,[20,21], similar to the route to the pulmonary nodes of talc miners.[22] Lymph nodes of the pelvic region include several anatomic sub-classifications (inguinal, iliac, and paraaortic), with the common theme that they may receive lymphatic efferents from pelvic organs such as the ovaries and perineum and/or secondarily from other lymph nodes in the area. Ovarian carcinoma, especially serous, tends to metastasize early (when just one or two nodes are involved) to paraaortic nodes.[23] Full discussions of the lymphatic drainage/anatomy of the pelvic region are available in the literature.[20,21] Lymph nodes are often sampled during gynecologic surgery for tumor staging and assessment for metastatic disease. However, additional examination of these nodes for talc, especially in settings where genital exposure is known to have occurred, would add insight as to the ability of talc to migrate and lodge within pelvic tissues.

This study supports earlier observations that talc particles, from perineal exposure, can and do migrate to pelvic lymph nodes. Material with the microscopic and spectral features of talc was clearly demonstrated within the lymph node parenchyma in most of our cases, as scattered birefringent particles in the general size range 1–10 μm. Sometimes the material was visible within nodal macrophages, lending strong credence to a lymphatic migration route. Similar particles were also found in the fibroadipose tissue adjacent to lymph nodes, where they may have arrived via the lymphatic system, but more likely resulted from visibly present surface contamination pushed into the underlying fibroadipose tissue.

Our study took the additional critical step of comparing the light microscopic data to SEM digestion data, thereby going beyond the earlier study by Heller et al.[17] in scope, in addition to examining lymph nodes rather than ovaries. Like that earlier paper, we found high talc particle burdens in some digested samples. But because these correlated with contamination scores, we believe that the digestion counts are not fully reflective of clinically relevant talc exposure or its migration in the tissues. Instead, they are influenced by contamination, such as talc introduced by non-surgical gloves used for handling tissue and in the general lab environment during tissue collection and processing in the pathology laboratory. Thus, tissue digestion should not be regarded as a reliable quantification method for talc or contaminants of talc, especially where the collection and processing steps have not been rigidly controlled from the start. The correlation of contamination scores with counts of birefringent particles in fibroadipose tissue suggests that particles adherent to the surface (through contamination) may be pushed into the soft fibroadipose tissue, since it is typically the most peripheral type of tissue, with the nodal tissue usually deeper and encapsulated with a fibrous tissue capsule. The highly variable talc burdens found by digestive analysis and SEM, spanning approximately three orders of magnitude, are consistent with contamination influence, since the latter would be expected to vary considerably between procurement environments. However, this could also be observed in the range of burdens seen in a clinically exposed population with appropriate lab procedures/controls (Table 4).

Even though contamination played a role in total tissue counts, it was still the case that high talc burdens in the lymph nodes, when present, contributed to the SEM digestate results, hence producing the observed correlation between the two. Thus, it is likely that both contamination and clinically significant lymph node talc are reflected in the SEM digestate data. The main

problem in using digestion is that it likely raises the baseline for all patients and groups, thus potentially obscuring clinically significant differences, which would otherwise be observed if contamination were eliminated (as previously mentioned, Table 3 illustrates a robust demonstration of this effect).

By showing strong correlations between particle counts (polarized light microscopy) and *in situ* SEM analysis, the second part of our study demonstrated that the latter alternative is a better method of talc assessment than digestion, because the anatomic landmarks are preserved and surface contamination is not incorporated into the general talc count, as it is with tissue digestion. In combination with other parts of our study, this aspect also showed that the birefringent material in the lymph node tissue, is the clinically significant component related to talc exposure. Surface contamination can still be present, and our demonstration of talc on the surfaces of cases 1B and 7A by *in situ* SEM lent support to the conclusions from the first (digestion) part of the study.

A major strength of our study was the correlative light microscopic and SEM/EDX data for each case, with examination of anatomic locations in the former. This provided a key perspective in the evaluation of the talc burden data that a digestive study alone would not have given. In fact, this study demonstrates the broader principle that correlative histologic review is important in many areas of pathology – especially where digestion procedures are performed, and where the study of anatomic landmarks are needed to complement data from the latter. This is because the tissue is compartmentalized histologically, with different functions and significance for each component, a fact not always recognized by those who digest tissue routinely and use the resulting product completely in analyses such as Western blotting or mutational assays.[24]

Unfortunately, as part of our study, we were not able to also do *in situ* SEM/EDX on the intact tissues used for digestion in the first group of cases (22 patients). However, by showing that birefringent particles within lymph nodes were strongly correlated with the demonstration of talc inside the nodes by *in situ* SEM/EDX, the second part of our study filled that role, and thus 1) material in lymph nodes is likely reflective of the clinical exposure, 2) in this clinical setting and given our results, a substantial proportion of this birefringent material is likely to be talc, 3) surface contamination is common, and so with *in situ* SEM, it is important to discern the anatomic landmarks, and avoid analyzing surface particulates (as shown by our direct demonstration of talc on the surfaces of cases 1B and 7A in our auxiliary study to the cases in Table 4).

In addition to talc, much other commonly found birefringent material, such as that described in the Results section for the SEM analysis, is likely nonspecific particulate material which finds its way into the perineum through general living and hygiene practices. Another important point is that seeing particles by *in situ* microscopy, both light and SEM, requires a relatively large amount of material distributed within the tissues in order to find it. As a demonstration of this principle, Roggli and Pratt[25] showed that finding one asbestos body in a tissue section was indicative of at least 100 fibers per gram of tissue. The calculations we used to estimate particles/cm^3 of tissue volume (Table 1), starting with a count of birefringent particles in tissue sections, illustrate a similar principle.

In the long-studied and debated association between talc exposure and ovarian cancer, our study provides additional evidence that talc may enter pelvic tissues and ultimately be detected and measured in regional lymph nodes, and this relationship became especially strong when clinical use data was considered and surface contamination was corrected for statistically. This adds perspective to the known migratory capabilities and overall biological role/impact of talc. For some of the more heavily exposed cases in the second part of the study, we noticed that the large majority of birefringent material was localized in a single node, among several present on a given slide. This suggested that pelvic drainage/migration pathways for talc may be very specific, and focused on one or relatively few nodes as an endpoint – perhaps consistent with the concept of sentinel nodes in oncologic surgery.[26]

Our findings also suggest that in patients with ovarian cancer, clinicians may want to make broader inquiries into the past and present use

of talc by their patients. Similarly, pathologists may wish to pay greater attention to sampled regional lymph nodes. In addition to the usual study of these nodes for metastases, they may wish to examine macrophages more closely for exogenous particles including by polarized light. A positive finding may trigger clinical inquiries about exposure where it was not previously suspected. Our findings yield important insights as to the ability of talc to migrate to nodes, and under what conditions its identification in nodes and tissues is clinically meaningful and when not.

In conclusion, talc contamination of the surface of surgical pathology specimens is common. Exposure (such as perineal application), whether known clinically or not, often results in significant deposition of talc in the tissues. Correlative light microscopy is needed to assess the possibility of lab contamination, and to determine if talc is truly present in clinically meaningful locations in lymph nodes or other tissues.

Declaration of Interest Statement

The authors declare the following competing financial interest(s): JJG, DC and WW have served as consultants and provided expert testimony in talc and other environ mental litigation. SM, YF, RS, MK, and LS report no conflicts of interest.

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Exhibit 53

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ORIGINAL ARTICLE

Does long-term talc exposure have a carcinogenic effect on the female genital system of rats? An experimental pilot study

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Abstract
Objective In several studies, the prolonged exposure to talc has been associated with development of ovarian cancer. However, some studies have advocated contrary views. The present study aims to investigate histopathological changes and whether long-term talc exposure is associated with potential carcinogenic effects on the female genital organs of rats.
Materials and methods The present study was conducted at Dumlupinar University Medical Faculty and a total of 28 Sprague–Dawley rats were included. The experimental animals were allocated into four groups having seven rats each. Groups 1 and 2 served as controls, where the rats in Group 1 did not receive any intervention and Group 2 received intravaginal saline. Groups 3 and 4 received intravaginal or perineal talc application, respectively. Talc was applied for 3 months on a daily basis. Histopathological changes in the peritoneum and female genital system were evaluated. For statistical analyses, Fisher's exact test was carried out using SPSS.
Findings In both the groups exposed to talc (Groups 3 and 4), evidence of foreign body reaction and infection, along with an increase in inflammatory cells, were found in all the genital tissues. Genital infection was observed in 12 rats in the study group and 2 rats in the control group. Neoplastic change was not found. However, there was an increase in the number of follicles in animals exposed to talc. No peritoneal change was observed. In the groups not exposed to talc, similar infectious findings were found, but there was a statistically significant difference between the groups (Groups 1 and 2 vs. Groups 3 and 4, $P > 0.05$). Neoplastic change was also not observed in these groups. Four groups were compared in terms of neoplastic effects and infections. In Groups 1, 5 rats were normal, two developed vulvovaginitis and endometritis with overinfection (in both ovaries), and one developed salpingitis (in both fallopian tubes), that is, infection was found in a total of two rats. In Group 2, only one experimental animal had endometritis. All the animals in Groups 3 and 4 developed infections.
Conclusions Talc has unfavorable effects on the female genital system. However, this effect is in the form of foreign body reaction and infection, rather than being neoplastic.

Keywords Talc · Ovary · Endometrium · Vulva

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Introduction

Various factors have been cited in the development of genital cancers. Many studies have been conducted regarding the potential role of talc in ovarian cancer and previously it had been widely accepted as an important etiological factor [1–4]. In recent years, some studies (meta-analysis) have been averse toward this concept [5, 6]. Asbestos is a well-known carcinogen, and described as having a particular role in the development of pleural and peritoneal mesothelioma [7]. Its association with ovarian cancer has also been demonstrated in several studies [8–10].

Talc and asbestos are both silicate minerals. Minerals are classified according to their anionic structure, and its subclasses are defined by chemical composition or structure. Classes and subclasses can be further divided into mineral groups on the basis of atomic structure and chemical similarities. Talc is a magnesium silicate hydroxide, characterized by water molecules trapped between silicate sheets, which belongs to the silicate subclass phyllosilicate and the clay group montmorillonite/smectite. The three other major phyllosilicate clay groups are kaolinite/serpentine, illite, and chlorite [5].

Asbestos is the generic or commercial name for six naturally occurring fibrous minerals including amosite, chrysotile, and crocidolite, which are used in industrial applications, and the fibrous varieties of tremolite, actinolite, and anthophyllite. Asbestos is morphologically distinct from talc and belongs to different silicate mineral groups and subgroups. The carcinogenic effects of asbestos have been extensively studied and documented in medical literature [10, 11]. It is clear that the morphologic structure of serpentine asbestos and the fibrous form of amphiboles is responsible for their carcinogenic properties, much more than its atomic constituents [12]. In contrast, talc, which is a member of the montmorillonite/smectite group, rarely occurs in the asbestiform habit (a mineral's fibrous pattern of growth). Even asbestiform talc is not as carcinogenic as asbestos owing to its chemical and physical properties [5].

Although a number of studies have examined the relation between talc and ovarian cancer, its effect on other female genital system tissues have not been investigated. In addition, the carcinogenic effect of talc has not been ascertained. The present experimental study aimed to examine carcinogenic effects of long-term talc exposure on genital system of female Sprague–Dawley rats.

Materials and methods

Experimental animals

The experimental study was conducted at Dumlupınar University Medical Faculty and approved by the institutional ethics committee for animal experiments. A total of 28 Sprague–Dawley female rats weighing 200–250 g were used as experimental animals. Animals were kept in standard cages at room temperature under normal diurnal conditions (12 h day and 12 h night). Sufficient water and food intake was provided.

Groups

The experimental animals were assigned into four different groups having seven rats each. Group 1 served as control and did not receive any intervention. Group 2 also served as control and 0.5 ml of saline was intravaginally administered to these animals. Groups 3 and 4 were study groups and received intravaginal and perineal talc application (100 mg in 0.5 ml of saline), respectively. Talc with saline was given in aerosol form to the animals; dust form was not applied. However, this application can be optimally intravaginal. Talc application was done daily for 3 months. At the beginning of the study, cervicovaginal smear samples were obtained from each animal between 08:00 and 09:00 a.m.

Histopathological examination

At the end of the experiment, the animals were sacrificed under ether anesthesia by drawing blood from their hearts thus inducing hypovolemic shock. All the internal and external genital organs (vulva, vagina, uterus, fallopian tubes, and ovaries) were surgically removed and placed in 10% formaldehyde solution. The samples were examined for the changes in the peritoneum and female genital system. Hematoxylin and eosin (H&E) staining and light microscopy was used in the histopathological examination. For statistical analysis, Fisher's exact test was performed using the SPSS software. A P value <0.05 was considered significant.

Findings

Baseline smears revealed the presence of vaginitis in only two experimental animals, whereas findings were normal for the remaining 26 rats. One of the rats detected with vaginitis belonged to the study group and one of them belonged to the control group. At baseline, the mean weight of all rats was 226 ± 24 g (average weight of study groups was 228 ± 18 g, control groups was 224 ± 30 g) and the corresponding figure at the end of 3 months was 240 ± 20 g (229 ± 17 g study groups, 251 ± 23 g were found in the control groups) showing no significant change.

Foreign body reaction, findings of infection, and increased number of inflammatory cells were found in all groups exposed to talc (Groups 3 and 4). No neoplastic

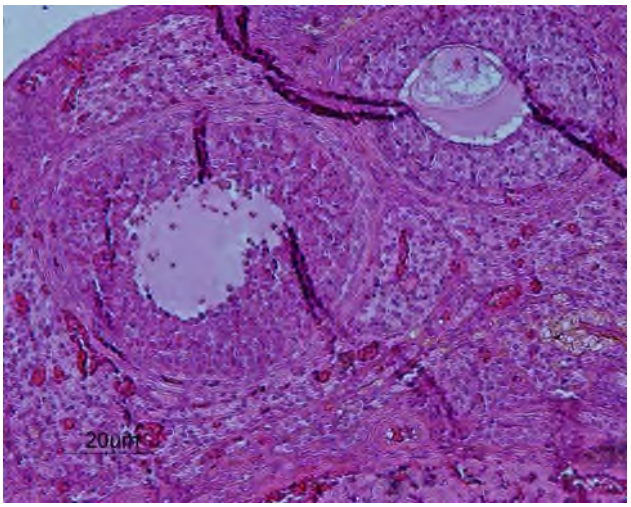

Fig. 1 An increase in the number of follicles was observed in the ovaries

change or peritoneal change was detected. Moreover, an increase in the number of follicles was observed in the ovaries of all animals in the control and study groups. (Fig. 1)

The four groups were compared in terms of neoplastic effects and infections. In Groups 1, 5 rats were normal, two developed vulvovaginitis and endometritis with over infection (in both ovaries), and one developed salpingitis (in both fallopian tubes). Infection was found in two rats. In Group 2, only one experimental animal had endometritis. In Groups 3 and 4, all the animals developed infection. Animals in Group 3 developed the following pathologies: vulvovaginitis ($n = 5$), endometritis ($n = 6$), pelvic infection ($n = 4$), ovarian infection (a total of 7 ovaries in 3 animals), and salpingitis and tubal occlusion ($n = 8$). In Group 4, all seven rats developed vulvovaginitis; furthermore, four developed endometritis, five developed pelvic infection, four developed ovarian infection (in eight ovaries), and two developed salpingitis and tubal occlusion (one unilateral and one bilateral). The analysis using Fisher's exact test showed positive correlations between following groups: Group 3 and Group 1, Group 3 and Group 2, Group 4 and Group 1, and Group 4 and Group 2 ($P < 0.05$). Other pairwise comparisons did not reveal significant results ($P > 0.05$) (Table 1).

Conclusions

The preliminary results show that in 28 rats from the 4 groups, talc had unfavorable effects on the female genital system. However, this effect seems to be in the form of foreign body reaction or infection rather than a neoplastic change. The results of previous studies are in favor of a neoplastic effect, particularly on the ovaries. However, more experimental and clinical studies are warranted to reach firm conclusions. In the study and control groups used in this research, an increase in follicle number was also observed. It should be emphasized that other environmental factors may have role in these effects. Therefore, the effect of different study conditions should also be investigated in detail.

Declaration

The present study was designed and conducted by Nadi Keskin MD, with the contributions and approval of Prof. Halil Saygili MD, an academic member of the Istanbul Medical Faculty, Istanbul University, as an extended study of 'Histopathological Changes Induced by talc Exposure in Rats' [9] previously published in 'Endokrinolojide Yönelisler Dergisi' (2005; volume 14, number 4). The language of this manuscript was edited by SPi Professional Editing Services (http://www.prof-editing.com/index.php).

Table 1 Groups of histopathological changes observed in the genital system of experimental animals

	Normal	Vulvovaginitis	Endometritis	P ID	Findings of ovarian infection ($n = 2 \times 7 = 14$)	Salpingitis and tubal occlusion ($n = 2 \times 7 = 14$)	Neoplastic changes	Preneoplastic changes
Group 1 ($n = 7$)	5	2	0	1	1 (2 ovaries)	1 (2 fallopian tubes)	0	0
Group 2 ($n = 7$)	6	0	1	0	0	0	0	0
Group 3 ($n = 7$)	0	5	6	4	7 ($2 \times 3 + 1$)*	8 (2×4)	0	0
Group 4 ($n = 7$)	0	7	4	5	8 (2×4)	5 ($2 \times 2 + 1$)**	0	0

Statistical comparisons of the groups were done by using Fischer exact test. Following positive correlations were found between groups: Group 3 and Group 1, $P = 0.021$, $P < 0.05$; Group 3 and Group 2, $P = 0.005$, $P < 0.05$; Group 4 and Group 1, $P = 0.021$, $P < 0.05$, Group 4 and Group 2, $P = 0.005$, $P < 0.05$. Other comparisons did not reveal statistically significant results ($P > 0.05$)

Group 1: control group with no intervention, Group 2: control group receiving intravaginal saline administration, Group 3: study group receiving intravaginal talc application, Group 4: study group receiving perineal talc application

* In this group, one rat had infection findings in only one ovary. For the remaining, both ovaries were involved, ** In this group, one rat had infection findings in only one fallopian tube

Discussion

Various etiological factors have been cited for genital cancers. A number of studies on talc have been conducted to investigate its effect on the development of ovarian cancer, the most fatal among these malignancies; it is commonly accepted as an important etiological factor for this cancer [1–4]. In recent years, some studies (meta-analysis) have been averse to defend this concept [5, 6]. Asbestos is a well-known carcinogen that has a particular role in the development of pleural or peritoneal mesothelioma. It has also been associated with ovarian cancer [8–10]. However, because of differences in the structure of talc and asbestos, the carcinogenic effect of asbestos has also been examined.

Talc is an important industrial material, because of its resistance to electricity, heat, and acid. Therefore, it is widely used in plastic surfaces, especially in surgical gloves, various plastic apparatus, and gynecologic services, and women are commonly known to use it for sanitation purposes. Other applications for talc include contraceptive diaphragms and condoms [5], and the treatment of pleural effusions and pleurodynia [13–15].

Surveys on the hygienic practices of women and talc application on the perineum, animal experiments, and clinical trials are among the studies that investigate the carcinogenic effects of talc [16, 17]. Since the first study showing an almost twofold increase in the risk of ovarian cancer with any perineal talc use [3], most case-control studies have demonstrated positive associations with talc use [4, 18]. Not all of them have been statistically significant [19–21]. Several studies [21–23] did not find an overall association between any genital talc use and ovarian cancer. Some studies [21, 22, 24] have demonstrated statistically insignificant trends in risk with increased frequency of talc use, duration of use, and measures of "total lifetime applications," whereas other studies [19, 20] have not observed a statistically significant dose response. With regard to histologic subtypes, a recent study by Cramer et al. [24] observed the greatest risk of talc use in invasive serous cancer; however, other studies have found increased risks for endometrioid cancers [4, 21], serous cancers [25], and invasive cancers of all subtypes [4]. Because serous cancers which account for over half of all invasive ovarian cancers, most resemble mesotheliomas, it could be hypothesized that this subtype may be most likely associated with talc use.

There have been few studies [26, 27] of talc exposure in animals and these studies have not demonstrated an increase in ovarian cancer among animals subjected to continuous talc exposure. These data should be interpreted cautiously because there are important anatomic and physiologic differences between rodents and humans. In animals, talc is often administered at high dose via aerosol exposure [26].

Some studies found a positive relation between ovarian cancer and perineal talc application. Cramer et al. [3], Rosenblatt et al. [28], and Chang et al. [4] have reported relative risks of 1.92 (%95 CI 1.3–2.9), 2.4 (%95 CI 1.1–5.3), and 1.42 (%95 CI 1.08–1.86), respectively. This shows an increased risk correlation between the use of talc as a cosmetic and ovarian cancer, but this increased risk is not significant. Studies by Cramer et al. describe the relationship between talc and ovarian cancer; thus, "Our studies have suggested an increased risk for ovarian cancer associated with the use of talcum powder in genital hygiene, but the biologic credibility of the association has been questioned."

In a meta-analysis, Huncharek implied that earlier epidemiological studies suggest an association between perineal cosmetic talc use and increased risk of epithelial ovarian cancer. This meta-analysis was performed to evaluate such an association. Available observational data do not support the existence of a causal relationship between perineal talc exposure and an increased risk of epithelial ovarian cancer [5]. Selection bias and uncontrolled confounding may account for positive associations observed in prior epidemiological studies. In addition, in a review, Muscat implied that talc is not genotoxic. Mechanistic, pathology, and animal model studies have not found evidence of a carcinogenic effect. In summary, these data collectively do not indicate that cosmetic talc causes ovarian cancer [6].

Talc has been used routinely in humans in the treatment of pleural effusions, where talc is directly applied to the human pleura. Long-term follow-up studies of humans undergoing this procedure have not shown a single case of malignancy induced by talc [13–15].

Animal data in relation to talc toxicity are important: Wagner et al. [29] used Italian talc in experimental animal study; Italian talc has been tested on rats using three routes, intra-pleural inoculation, inhalation, and ingestion. Groups exposed to superfine chrysotile asbestos and untreated controls were included for comparison. In all the experiments, animals were allowed to live out their lives. The intra-pleural inoculation of talc produced no mesotheliomas in contrast to 18 produced by the chrysotile asbestos. After ingestion, one leiomyosarcoma occurred with Italian talc and one with chrysotile asbestos. Whether these tumors are a consequence of the feeding is uncertain. The inhalation studies demonstrated that with equal dosage, talc can produce a similar amount of fibrosis as asbestos. However, the chrysotile-exposed rats developed lung adenomas, adenomatosis, and an adenocarcinoma, whereas the only lung tumor seen in animals exposed to talc was a small adenoma, which may have been an incidental finding. In another experimental animal study [30], 256 Wistar rats received a single injection of crocidolite into the right pleural cavity to induce mesotheliomas. Subsequently, they

were given right intra-pleural injections of BCG, crystalline silica, talc, carrageenan, or saline (as a control). There was no significant change in the mesothelioma rate in the rats exposed to BCG, silica or talc, but there was a threefold increase in mesothelioma incidence in the group injected with carrageenan.

Wagner, Huncharek, Hill, Muscat, and others hold that talc is not carcinogenic. Also in this study, carcinogenic effect of talc has not been determined either in any female rat genital system tissue or in ovary tissue.

In an experimental study on rats, Henderson et al. [31] demonstrated the presence of talc in the ovaries following vaginal talc application. Similarly, in the study of Egli et al. [32], carbon particles reached the fallopian tubes in 30–35 min. Therefore, talc disseminates to vulvovaginal region, endometrium, fallopian tubes, ovaries, and peritoneum after vaginal examination, raising the possibility of changes—even preneoplastic or neoplastic—due to talc exposure following perineal or vaginal talc application. Langseth et al. [33] are skeptical about the association between perineal talc application and ovarian cancer. In their article, Langseth et al. expressed that the origin of ovarian cancer is multifactorial, especially breast cancer patients who are BRCA 1 and BRCA 2 gene carriers, who have increased the risk of ovarian cancer. Therefore, the role of environmental factors such as talc and asbestos in cancer formation is suspected of being over stressed.

Salazar et al. examined histological changes in BRCA 1 gene positive women who underwent prophylactic oophorectomy because of their high inherited risk of ovarian cancer, and reported the following findings: increased follicular activity, hyperplasia of corpus luteum, hilar cell hyperplasia, pseudostratification of superficial epithelium, superficial papillomatosis, cortical stromal hyperplasia, and hyperkeratosis. These investigators defined increased follicular activity, hyperplasia of corpus luteum, and other findings as preneoplastic phenotypes in ovaries with high risk [34]. If talc application has a carcinogenic effect on ovaries, this study would also obtain similar findings. In the present study, experimental animals exposed to talc both via vaginal and perineal application for 3 months did not show the abovementioned changes except for an increased number of follicles. Although both the control and study groups showed an increase in follicle number, thus it was not attributed to talc application; however, the increase in follicle number could not be explained as well. Therefore, this study did not demonstrate an association between talc application and peritoneal/ovarian cancer.

In both groups exposed to talc (Groups 3 and 4), evidence of foreign body reaction and infection along with an increase in inflammatory cells were found in all the genital tissues. Muscat comments on this [6]: “Given the dissimilarities between talc and asbestos with regard to their fibrous shapes, the weak but increased associations in the epidemiologic studies could be attributed to other mechanisms assuming that the statistical associations are unbiased and not due to confounding. Asbestos fibers in the lung initiate an inflammatory and scarring process, and it has been proposed that ground talc, as a foreign body, might initiate an inflammatory response [35]. Pelvic inflammatory diseases, however, such as endometritis, peritonitis, tubo-ovarian access formation, and salpingo-oophoritis have in general not been associated with an increased risk of ovarian cancer.”

Similar to Muscat’s comments, this study also demonstrated unfavorable effects of talc on female genital system; however, it was in the form of foreign body reaction, infection, or increased adhesions, rather than neoplastic. In addition, the authors of this study believe that talc may have caused foreign body reaction, infection, or increased adhesions, which should be important for infertile patients.

Endometrial cancer is the most common genital malignancy among women. Unbalanced estrogen levels are being blamed for its etiology [36]. Various physical and chemical factors have also the potential to initiate preneoplastic and neoplastic stimulus [37]. A literature search did not reveal any study examining such an effect of talc on the endometrium. However, this study could not demonstrate any preneoplastic or neoplastic effect of talc on endometrial tissue.

Etiological factors for vulvar, vaginal, and cervical cancers have been largely discovered; in particular, HPV (Human Papilloma Virus) has been identified as an etiological factor [38]. In addition, physical and chemical factors have also been held responsible. However, talc is not counted among these factors and a clinical study examining such an effect does not exist. None of the rats in this study showed an evidence of such an effect.

Talc application has unfavorable effects on female genital system, particularly on ovaries and fallopian tubes. This usually manifests itself in the form of tissue injury, macrophage infiltration, and an increased rate of infections and development of adhesions [8, 39–41]. Holmdahl [42] emphasized the important role of talc in the development of adhesions after intraperitoneal surgery. Merritt et al. [8] reported an increase in chronic pelvic infections following perineal talc application. Ellis et al. [43] also emphasized the unfavorable effects of talc spilling from surgical gloves. In the present study, compared to the controls, a significantly increased rate of infection was found among the rats exposed to talc, which was particularly prominent for endometrial tissue, uterine tubes, and pelvic peritoneum. These tissues exhibited epithelial tissue injury, macrophage infiltration, and adhesions. Tubal adhesions are important in the context of infertility. In addition, the high rate of vulvovaginitis may have important implications for patients undergoing frequent gynecological examinations and for

immunocompromised patients attending outpatient gynecological oncology clinics.

In conclusion, the present study demonstrated unfavorable effects of talc on female genital system in the form of foreign body reaction, infection, or increased adhesions, rather than neoplastic. Moreover, the authors believe that talc may have a stimulating effect on ovaries, which should be further investigated particularly in infertile patients. However, the authors of this study highlight the fact that other environmental factors may have role in the increased follicle number presented by the control group. Therefore, separate intensive studies in the series, to demonstrate the effect of talc on the ovary should be considered.

Conflict of interest statement None.

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Exhibit 54

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Principles for the safety evaluation of cosmetic powders

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ABSTRACT

Consumer exposure to cosmetic (personal care) products is mostly by dermal contact, however additional considerations with regards to potential inhalation exposure from some cosmetics, such as sprays and powders, may be needed for a robust and reliable safety assessment.

To get a deeper understanding of the exposure to airborne particles and droplets during product application, a team of international experts was founded under the umbrella of the European Association of the Cosmetic Industry "Cosmetics Europe" (CE) in Brussels. This expert team has worked out a pragmatic strategy how small and medium sized enterprises (SMEs), but also relevant authorities, could handle the safety evaluation of cosmetic powder products. Sufficient information on the aerodynamic diameter of sprayed droplets and here specifically of airborne particles is essential in addition to knowing the exposure after typical product application. The current article is focused on the determination of inhalation exposure to solids, and the derivation of safe exposure levels for cosmetic powder products found in the market. The principles described herein are very similar to spray products as published earlier and should be applied in a similar way (Steiling et al., 2014).

Prediction models for the best estimate of inhalation exposure, developed with data from computer simulation programs, individual real-time measurements or finally by experience from the market were introduced and applied. Safety assessment approaches for exposure from powder spray products were developed and have been already considered in regulatory guidelines like the EC Cosmetics Regulation.

1. Introduction

Although dermal contactis the prominent exposure route for most cosmetic products, cosmetic sprays and powders have to be evaluated regarding inhalation exposure as well. This guidance provides princi ples for the safety assessment of cosmetic powders under typical use conditions, which are based on previous publications on the assessment of sprayed cosmetic products (Steiling et al., 2014; Rothe et al., 2011). The main goal is to provide insights for the assessment of inhalation exposure and to share best practices and basic guidelines for the safety assessment of cosmetic powder products without the need to go into standard inhalation toxicity testing.

Like the droplet size of liquid aerosols, the particle size of a solid material, their distribution and density in a room is essential for the exposure estimation of powders in the respiratory tract and key for a proper and reliable safety assessment. Common calculation (*in silico*) models for inhalation exposure are assessed for optimization and har monization when applied in safety assessment, for consumers, profes sional users such as hairdressers and during product manufacturing. The challenges and benefits of the exposure calculation models will be outlined. The measurement of particle size distribution during product use is also introduced. Furthermore, relevant factors to consider when producing and/or formulating a cosmetic powder such as particle size, binding materials and milling procedures will be discussed.

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2. Distinct types of cosmetic powders

Cosmetic powders have been used since ancient times (e.g. for make up). Such products are typically marketed as loose (flow) powders or compact (pressed) powders. They are used to provide adhesiveness, slipperiness, adsorbance, smoothness and the bloom effect they provide to the skin or hair (Farber, 1972; Moussour et al., 2016).

In general, cosmetic powder products are similar and standardized regarding their ingredient composition. Essentially, they contain ingredients like fillers (e.g. talc, kaolin, calcium and magnesium carbonate, metallic stearates, silica and silicates), colors (e.g. pigments, lakes, mica, bismuth oxychloride), preservatives, perfume and binding agents (e.g. mineral oils, fatty esters, lanolin and derivatives, gums, emulsifying agents). The composition of ingredients, along with their particle size and physical properties (e.g. adhesive character), has an impact on the technical quality of the final powder formulation (Farber, 1972; Patel and Frischman, 1986).

Pearls, mainly mica based ingredients with titanium dioxide and colors, are widely used in powders for their coloring properties and visual effects. Milling processes, binder content and compression define the final particle size in a powder, but the often seen agglomeration of such particles during manufacturing fosters the increase of the final particle size in the cosmetic product (Scientific Committee on Consumer Safety (SCCS, 2012a; Farber, 1972).

2.1. Loose (flow) powders

Loose (flow) powders include finishing powders, foundations, perfumed body powders, cheek powders, eye shadows, dry shampoos or others. As already mentioned, the composition of loose powders is generally standardized, e.g. a dry shampoo typically contains two or more powdered ingredients to absorb the scalp sebum (oil). Particle sizes in loose powders are generally smaller compared to those of compact (pressed) powders and triggers the performance of the powder product (Bennett, 2017). To minimize potential inhalation exposure dust free powders are in the market (Nilsson et al., 2016). This dust free status is attained by the virtue of containing some oily ingredients (Marrs, 1990).

Body powders, with the capability to absorb moisture from the skin surface, e.g. in the nappy zone of babies (Barel et al., 2014), are usually marketed as loose powders in a box or in a shaker. As they are typically stored in the bathroom, often under humid conditions, they are protected against agglutination, e.g. by using a combination of absorbent and non absorbent silica.

Loose powders, in contrast to baby powders, are typically applied with a puff, or make up brush.

2.2. Compact (pressed) powders

Compact (pressed) powders are dry powders compressed in cake form (Schlossman and Feldman, 1971). Such powders are usually applied to the body, face or eye area with a puff (fluffy sponge) or a brush. As make up, they are the most extensively used cosmetic items nowadays.

Pressed powders are of a similar composition to loose powders, but contain a significantly higher amount of binders (Oberacker, 2012). During the manufacturing of a pressed powder foundation, the use of a jet mill is preferred as it results in unique spherical shaped particles. In contrast, a hammer mill or micro pulverizer produce irregular shapes and larger particles. The higher level of binder contributes to the overall feel of the product during application and minimizes airborne particles during use (Braunagel, 2002).

3. Regulatory requirements for powders

3.1. REACH/MSDS

All cosmetic ingredients, including those which are specifically used in cosmetic powders, have to fulfill REACH requirements as individual chemicals. For those produced in quantities exceeding 10 tons per annum and for those which are likely to be inhalable, i.e. potentially breathed in, based on their volatility, standard tests on acute inhalation toxicity are requested. Data of such tests and any classification according to GHS/CLP (Globally Harmonized System) which has been implemented in the EU in form of the Regulation (EC) No 1272/2008 on the classification, labeling and packaging of substances and mixtures CLP Regulation) will be available via the material safety data sheet (MSDS) (GHS, 2015; EU, 2008). Each individual hazard classification needs to be considered in the safety assessment of the cosmetic ingredients.

3.2. Occupational exposure limits

Apart from chemical substance specific regulations via REACH or product type specific regulations like the Cosmetics Regulation, occupational exposure limits (OEL) are defined for certain ingredients. Such OEL values have been developed as permissible maximum levels of exposure for healthy adult workers for eight hours work per day, five days per week. OELs are useful to ensure minimization of health concern during manufacturing or even when professionally used, e.g. in a hairdresser salon.

OELs, which are updated regularly, do exist for certain chemicals, but the values could differ in individual countries, mainly due to the use of different safety/uncertainty factors, e.g. talc (hydrated magnesium silicate) as a respirable dust (< 10 µm diameter (WHO, 1999)): UK OEL = 1 mg/m^3 in contrast to USA (National Institute of Safety and Health (NIOSH)), Austria and Switzerland with OEL = 2 mg/m^3. Beside such OELs, other permissible exposure levels to industrial chemicals do exist, e.g. the American Conference of Governmental and Industrial Hygienists (ACGIH) defined the TLV TWA (threshold limit value time weighted average) for respirable talc particles at 2 mg/m^3 for 8 h. For respirable, poorly soluble low toxicity particles, a general limit concentration of 5 mg/m^3 for an 8 hour workplace [TWA permissible exposure limit (PEL)] was set by the US Occupational Safety and Health Administration (OSHA). Per the German MAK Commission (Deutsche Forschungsgemeinschaft (DFG, 2017), the general threshold exposure limit applies to dust with a standard density of 1 g/cm^3, whose particles are not systemically bioavailable, i.e. dusts that are in insoluble (inert) or poorly soluble in biological fluids. The MAK exposure limit for such bio persistent granular dusts is 0.3 mg/m^3 for the respirable fraction (airborne material < 10 µm in aerodynamic diameter that can reach the alveolar region of the lung) and 4 mg/m^3 for the inhalable fraction (airborne material ≤100 µm in aerodynamic diameter that can be deposited anywhere within the respiratory tract). In cases of different densities, a related factor has to be applied.

As the above introduced occupational exposure scenario for solid particles (dust) is quite different and conservative compared to the typical consumer exposure during the typical use of cosmetic products like loose powders, OELs may serve as a first rough estimate for consumer exposure or professional use in a hairdresser salon in safety assessments of powder products.

3.3. Cosmetic use

In compliance with the EU Cosmetics Regulation (EC) No. 1223/2009 (EU, 2009) cosmetic powders, like other cosmetic products, must be assessed for their safety before being placed on the market for the intended use and foreseeable misuse. The SCCS Notes of Guidance (the European expert panel of the EU commission: Scientific Committee on

Consumer Safety and their Safety Evaluation) for the testing of cosmetic ingredients and their safety evaluation outlines the key elements that should be considered (Scientific Committee on Consumer Safety (SCCS, 2015). Therein, it is stressed that all relevant routes of human exposure need to be considered (Scientific Committee on Consumer Safety (SCCS, 2015). Consequently, depending on the type of cosmetic product and the application form, in addition to dermal exposure, there may be non intended inhalation exposure (e.g. to a loose powder).

In contrast to the occupational scenario the intentional application of cosmetic products is quite different as both the exposure duration as well as the typical quantities of airborne particles is less prominent. Furthermore, the toxic potential of the ingredients used is significantly lower compared to general industrial chemicals, as all of them have to be approved as safe for the use in such consumer products.

Currently, no specific requirements for cosmetic powders exist in the EU Cosmetics Regulation 1223/2009, but there are restrictions for individual ingredients. As an example, talc is listed in the EU Cosmetics Regulation Annex III (list of substances which cosmetic products must not contain except subject to the restrictions laid down) with the pre cautionary warning requirement “Keep powder away from children’s nose and mouth”. Furthermore, there are special restrictions for the UV filters titanium dioxide and zinc oxide if the materials contain particles in the nano size range; as listed in Annex VI entry 27a and 30a (list of UV filters allowed in cosmetic products) as usage restriction of: “Not to be used in applications that may lead to exposure of the end user's lungs by inhalation”.

As in the EU, the US FDA (US Food and Drug Administration) re quires cosmetic products to be safe without setting specific require ments as to how such safety, specifically of powders, has to be assessed. US Regulations such as Proposition 65 in California have ingredient specific restrictions similar to those of the EU Cosmetic Regulation. Ingredients deemed to be of concern due to inhalation, e.g. titanium dioxide commonly used as a colourant or pearl in powdered cosmetics, have considerations, which must be met to ensure their safety when typically used in consumer products. The ingredient is assessed as ‘of concern’ if meets three criteria: being unbound, airborne and of re spirable size (OEHHA, 2011). These criteria were used in several safety evaluations applying the particle size of 10 µm as the cut off for the respirable fraction of airborne particles (IARC, 1996).

In addition to the mentioned official regulations, there are guidance documents such as those of the US Cosmetic Ingredient Review (CIR), which are useful in safety assessments. In 2015, the CIR issued a gui dance document on how to assess inhalation risks to cosmetic powders. The expert panel referred specifically to habitual human data published on talc powders in consumer products to estimate typical exposure scenarios. The estimated consumer powder exposure was found to be significantly lower by consumer products than the TWA limit for oc cupational exposure to respirable talc particles and poorly soluble low toxicity particles. As a rough estimate and for orientation, the CIR panel compared the above mentioned 8 hour TWA limit with a typical 1.23 minute exposure of a cosmetic talc based product in which the respir able airborne fraction in the breathing zone was estimated to be 2.03 mg/m^3. The calculated concentration for the respirable fraction of 0.0052 mg/m^3 is significantly lower than the occupational TWA limit. Based on this information the CIR concluded that loose powders do not contribute significantly to the overall chemical exposure to the con sumer during use (Cosmetic Ingredient review (CIR, 2015).

4. Data obtained from *in vivo* testing

Animal tests with finished cosmetic products have been prohibited in the EU since 2004. A similar testing ban came into force in 2009 for single application tests and since 2013 for all animal tests even for individual cosmetic ingredients (EU, 2009). Consequently, the safety assessor of cosmetic products must rely on historical data and literature information on animal responses from studies generated before the ban. However, industry is currently making a concerted effort to develop and validate a battery of alternative *in vitro* methods to cover specific aspects of inhalation toxicity responses (ITEM, 2017).

Similar to the oral and dermal contact to chemicals, sprayed sub stances and powders have to be safe in regard to their systemic toxicity and local compatibility in the respiratory tract and therefore adequately evaluated. The appropriate use of historical animal data (e.g. some times available via the ingredient specific MSDS) requests a certain understanding of such inhalation toxicity tests of the individual in gredients.

Soluble and bigger particles (> 10 µm) tend to be rapidly cleared from the respiratory tract, e.g. via the mucociliary escalator, and sub sequently swallowed (Krinke, 2000) and therefore particle accumula tion from one exposure to another is unlikely. These soluble, as well as larger insoluble particles are not expected to affect the deep lung but may need to be considered in terms of oral exposure and local effects in the upper respiratory tract. In contrast, smaller insoluble particles may not be sufficiently cleared during breathing but may deposited in the alveolar region of the lung and a time dependent accumulation of the exposed powder may occur during the study (resulting in a ‘lung overload’) with resultant impairment of particle clearance and particle mediated inflammatory response. Under ambient environment such circumstance could not occur. For particle size analyses, a mass based metric allowing direct comparison with mass based actual concentra tions is needed (OECD, 2009). Any miss dosing due to inappropriate particle size will result in inconclusive data and will compromise the validity of test data (Warheit et al., 2016). Possible local effects to the respiratory tract, due to short term exposure (acute toxicity) or re peated exposure over a longer period (sub acute or sub chronic toxi city) must be clarified as well. For both exposure scenarios, appropriate protocols for *in vivo* tests are available and standardized as OECD testing guidelines (TG). Other guidelines, like those of the EPA (En vironmental Protection Agency of the USA), FIFRA (Federal Insecticide, Fungicide and Rodenticide Act of the USA) or the FDA (Food and Drug Administration of the USA), do exist but are focused typically on dif ferent product types (Krinke, 2000). Under standard experimental conditions in inhalation studies, the test chemical is applied via nose only exposure to mice or rats to avoid leaking of substance landing on their fur (Barel et al., 2014). It is important to mention species specific anatomical characteristics of the respiratory tract and some significant differences between rodents and humans (Raabe et al., 1988). There fore, data of inhalation toxicity studies has to be correctly interpreted considering specific anatomic situation. For example, there is convin cing evidence that poorly soluble low toxicity particles deposed in the respiratory tract exert two unifying major modes of action (MoA), in which one appears to be deposition related acute, whilst the other is retention related and occurs with particle accumulation in the lung and associated persistent inflammation (Pauluhn, 2014). The latter, called “lung overload” after chronic exposure, could even initiate lung tumors (Relier et al., 2017). The human lung is about more than seven times more resistant to attaining a lung overload, due to a larger and higher number of human *versus* rat alveolar macrophages involved in the clearing process (Pauluhn, 2014). Consequently, in humans there seems to be no correlation between particle exposure (except fiber structured) and lung cancer, as has been demonstrated by epidemiological studies on workers exposed to poorly soluble particulates (Warheit et al., 2016).

Today, calculation models like the multiple path particle dosimetry (MPPD) model for particles do exist to predict the deposition under given human or rat exposure conditions, necessary for an adequate comparison. This then allows the safety assessor to translate the quantity of respirable material applied in the rodent study to the spe cific human lung situation (the so called human equivalent concentra tion principle). This is described as follows: the MPPD model is used to calculate the deposition fractions of particles in rodents and humans. Using the rodent values for deposition fraction (DF), tidal volume (TV),

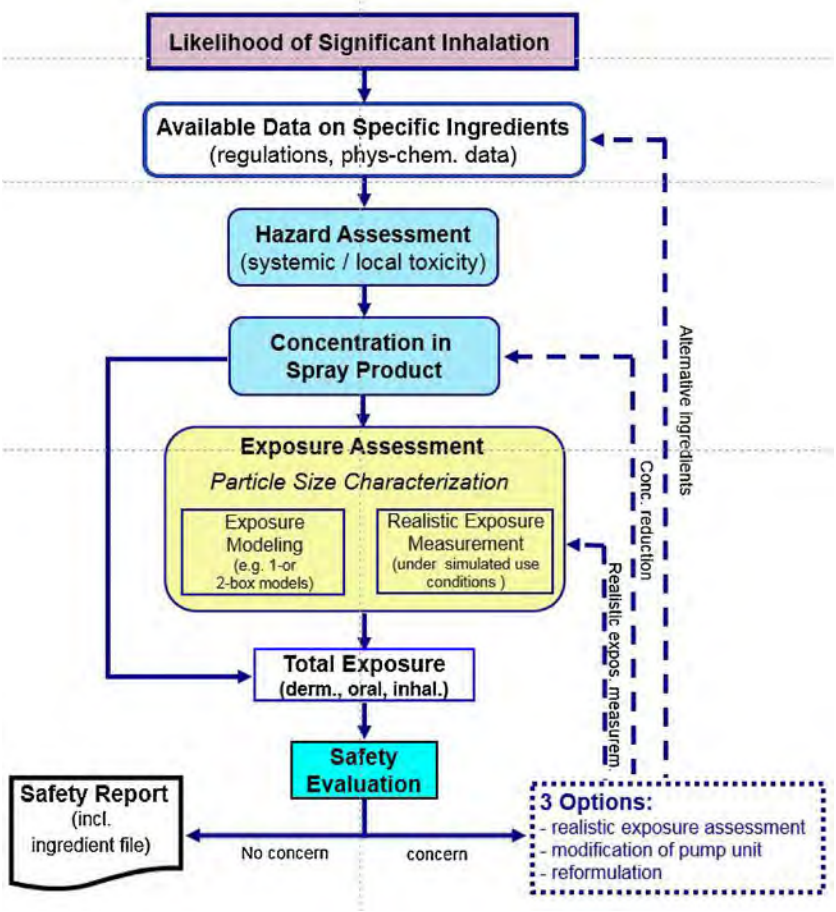


Fig. 1. Tiered approach for the inhalation safety assessment of spray products with different options for the exposure assessment (Scientific Committee on Consumer Safety (SCCS, 2015).

duration of exposure (T), breathing frequency (BF), alveolar surface area (SA) and the NOAEC (no observed adverse effect concentration) from a sub chronic inhalation toxicity study, the rodent deposited dose per alveolar surface area (DD_r) is determined, i.e. DD_r = (DF x NOAEC x TV x T x BF) ÷ SA. From this, the human equivalent concentration (HEC) of the rodent NOAEC is established, or back calculated, using the same parameters but by employing human values instead of rodent ones, i.e. HEC = (SA x DD_r) ÷ (DF x TV x T x BF). The HEC would be an absolute worst case as it assumes that the human exposure is to the same particles as to that of the rodent, and not considering the higher resistance of humans to attain a lung overload (see above). The HEC or point of departure (POD) or human derived no effect level (DNEL) the level above which humans should not be exposed could be then used in safety assessment (Kuempel et al., 2018).

4.1. Generation of small particles (dust) tested in vivo

As already mentioned above, the geometric dimension of particles (size, shape, density) determines their deposition and retention in the respiratory tract. However, in referring to particle size of airborne dust, the term "particle diameter" alone is an over simplification, since the geometric size of a particle does not fully explain how it behaves in its airborne state. Therefore, the most appropriate measure of particle size, for most occupational hygiene situations, is particle aerodynamic dia meter (d_{ae}), defined as "the diameter of a hypothetical sphere of density 1 g/cm^3 having the same terminal settling velocity in calm air as the particle in question, regardless of its geometric size, shape and true density" (WHO, 1999). The authors fully agree with this definition, but for practical application in safety assessment of cosmetic powder products the scientifically imprecise dimension “diameter” may be ap propriate.

As required by the relevant OECD TG, particles must be small en ough to be respirable in the standard experimental toxicity test system. For example, TG 39 (Acute 4 hour Inhalation) recommends that aero sols are generated with mass median aerodynamic diameters (MMAD) ranging from 1 to 4 µm with a geometric standard deviation (σg) in the range of 1.5 to 3. If the MMAD significantly exceeds 4 µm, further ef forts should be employed to reduce the MMAD down to the re commended range. To ensure equivalent chamber concentrations and particle size distributions, specific pre tests may be necessary. In prac tice, it can be difficult or impossible to generate such dimensions of respirable solids at the upper required concentration of 5 mg/L without encountering experimental shortcomings. These shortcomings are not limited to: a decrease in the respirable particle size fraction, increased fluctuation and variability in inhalation chamber concentrations ac companied by increased spatial inhomogeneities, and a divergence of nominal and actual concentrations. At very high concentrations, air borne dry powder and chemically reactive liquid aerosols (e.g. poly mers) tend to form conglomerates in the proximal nose causing physical obstruction of the animals’ airways (e.g. dust loading) and impaired respiration which may be misdiagnosed as a toxic effect (OECD, 2009).

There are a multitude of devices available that can be used to ensure the required fine airborne dust is generated. Just two such devices are the Wright dust feed mechanism (Wright, 1950) or a piston filled with a dry powder pressed against a rotating brush (Wolff and Dorato, 1997).

5. Safety evaluation of cosmetic powders

The safety evaluation of cosmetic powders is based on information from their individual ingredients, which may be classified according to CLP/GHS (EU, 2008; GHS, 2015), and on reliable exposure data. Before considering the details of the inhalation safety assessment of powdered cosmetic products, a brief introduction of the current approach for spray products is given.

5.1. Current approach for spray products

In recent years, the principle considerations for the safety evaluation of cosmetic spray products have been published (Rothe et al., 2011; Steiling et al., 2014) and adopted into EU Commission guidance via several revisions of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation (Scientific Committee on Consumer Safety (SCCS, 2012b, 2015, 2016). For each individual ingredient the physical chemical properties, data on its safety and regulatory status as well as the intended use level in the final product have to be evaluated to assess the hazard when it becomes inhaled. The hazard identification and the estimated likelihood of exposure when sprayed (hazard characterisation) are key elements for the safety evaluation of such sprayed products. A thorough understanding of realistic inhalation exposure of consumers may also be needed for a robust risk assessment. In a tiered approach (see Fig. 1), product exposure can be calculated in a first step by applying *in silico* exposure models or in a second step by measurement during simulated use as the most realistic approach (exposure assessment). In some situations where the risk assessment turns out to be unfavourable, the refinement of the exposure assessment, modification of spray characteristics (e.g. technical details of the spray can) or product reformulation may be required.

5.2. Powdered products

In contrast to liquids, where droplet maturation (a process where, due to solvent evaporation, a reduction in droplet size of the non volatile components of the formulation results) is important when the product is sprayed, airborne solid powders typically do not exhibit this phenomenon, but aggregation and/or agglomeration of particles is found. Such aggregation/agglomeration typically end up in a significant increase of the particle size. Following the schema given in Fig. 1, and prior to the final safety evaluation, the safety assessor should consider the following for the intended use of the powder (determination should be based on the final formulation and exposure to the specific product):

1) Will the powder reach the breathing zone, i.e. does the powder have the potential to form a dust cloud or atmosphere and therefore is there the potential for inhalation exposure? (Industry data indicate a low likelihood of dust cloud formation when pressed powders are applied with a brush (Wright, 1950).
2) Will the powder reach the breathing zone at relevant particle sizes, i.e. if a dust cloud or atmosphere can be formed, are respirable particles (< 10 µm) generated?
3) Will the powder reach the breathing zone at relevant doses, i.e. if a dust cloud or atmosphere can be formed and respirable particles are generated, what is the consumer exposure to the product?

Based on these questions, Fig. 2 represents a decision tree for the exposure assessment. The red box corresponds to the exisiting approach for spray products. The principles depicted in this decision tree are explained further in the following sections. It should be emphasised that possible incompatibilities to surfaces of the respiratory tract have to be assessed separately as not limited to the respirable fraction.

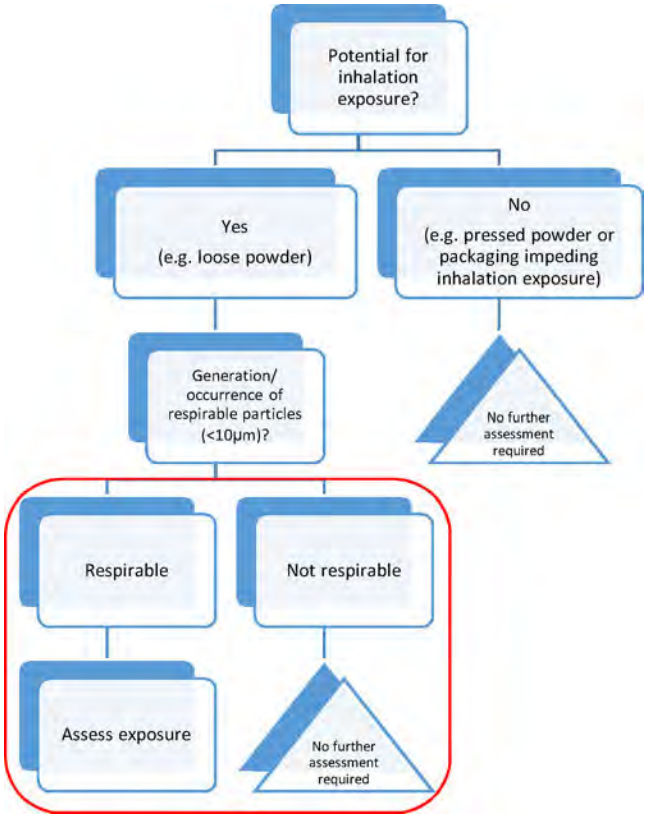


Fig. 2. Extended inhalation exposure assessment approach for cosmetic powder products.

6. Exposure data

6.1. Definition of dust

The WHO (World Health Organization) defines dust as "*small solid particles, conventionally taken as those particles below 75 µm in diameter, which settle out under their own weight, but which may remain suspended for some time*" The WHO also stated: *"In aerodynamic diameter terms, only about 1% of 10 µm particles gets as far as the alveolar region, so 10 µm is usually considered the practical upper size limit for penetration to this region."* (WHO, 1999). According to the "Glossary of Atmospheric Chemistry Terms" (Calvert, 1990) dust is *"Small, dry, solid particles projected into the air by natural forces, such as wind, volcanic eruption, and by mechanical or man made processes such as crushing, grinding, milling, drilling, demolition, shoveling, conveying, screening, bagging, and sweeping. Dust particles are usually in the size range from about 1 to 100 µm in diameter, and they settle slowly under the influence of gravity*.".

6.2. Dust cloud formation and the potential for inhalation exposure

The generation of a dust cloud or atmosphere during powder handling or application depends on its physical form, composition and the way it is applied. Compact (pressed) powders are unlikely to create a dust cloud or atmosphere due to the bonds formed between particles during the compression process, which provides coherence to the powder (Schlossman and Feldman, 1971). The usual application method of compact cosmetic powders (sponge, cotton wool patch, padded brush type implement or puff), associated with the small amount used per application, is unlikely to create a dust cloud or atmosphere of any significance, and therefore there is negligible inhalation exposure (Nazarenko et al., 2012a, b). In contrast, loose (flow) powders, which lack this particle cohesion, could generate such a dust cloud or atmosphere during product handling or application, and

therefore there is the potential forinhalation exposure. It should be noted, that larger particles (e.g. “aggregates and/or agglomerates”) are easily cleared by sedimentation.

6.3. Dustiness and the potential for inhalation exposure

Solid ingredients used in cosmetic powders can be inherently ‘dusty’ and when used in powders they may create a dust cloud or atmosphere simply through the removal of the lid from the storage container. Others may be hydroscopic, resulting in powders that readily agglomerate or become ‘sticky’ and consequently less dust generation is seen. When technically feasible and appropriate, the manufacturer may choose to produce granulated or pelleted products in order to reduce dustiness. Alternatively, a powder may be ‘dedusted’ to control generation of airborne material by the addition of other ingredients such as water or mineral oils (Marrs, 1990).

For classification purposes under REACH, the ‘dustiness’ of a material has to be evaluated (Lidén, 2006). Several methods exist for estimating the dustiness of industrial (Hamelmann and Schmidt, 2003; Lidén, 2006; Bach and Schmidt, 2008; Hauert and Radandt, 2009) or pharmaceutical powders (Boundy et al., 2006). The European Committee for Standardisation (CEN) issued a European standard for dustiness testing (EN 15051) in April 2006. This standard describes two methods, a rotating drum method (EN, 2013a) and a continuous drop method (EN, 2013b). The former is developed for mixing powders, the latter is for a bulk container being emptied out. These methods allow the classification of a powder regarding its dustiness as either very low, low, moderate or high risk for inhalable (airborne material ≤ 100 µm in aerodynamic diameter that can be deposited anywhere within the respiratory tract) and respirable (airborne material < 10 µm in aerodynamic diameter that can reach the alveolar region of the lung) dust fractions (Bach and Schmidt, 2008). Such measurement could also be employed to establish the dustiness of a finished powdered cosmetic product as a potential screening test for inhalation exposure risk.

Information on the dustiness of individual ingredients, provided in the corresponding MSDS or technical specifications, could be useful to provide a weight of evidence with respect to the potential dustiness of the final powdered cosmetic product. If this weight of evidence results in concerns of dustiness for the finished product, then this could be screened further, as described above.

The impact of dustiness on exposure can be significant. In a study characterising hairdresser exposure to airborne particles during hair bleaching, persulfate levels were measured in the breathing zone of hairdressers while using a standard bleaching powder and a reduced dust powder. The standard powder produced an average persulfate level of 26 µg/m^3, while the reduced dust powder level was only 11 µg/m^3 (Nilsson et al., 2016). This bisection of the air concentration means a reduction in exposure of more than 50%.

6.4. Particle size and particle size characterization

If a cosmetic powder has the potential to generate a dust cloud or atmosphere, then there is the potential for inhalation exposure during handling or application. Knowledge of particle size and their allocation to the three defined fractions (Fig. 3) becomes the next important factor for an exposure assessment. A predominance of larger particles would be toxicologically preferred as this would minimize inhalation exposure, foster increased clearance from the respiratory tract and ultimately reduces deep lung (alveolar) deposition/exposure. For example, particles greater than 30 µm would normally be captured in the nasopharyngeal region and lost through sneezing or nose blowing, while particles between 10 and 30 µm would tend to deposit in the tracheobronchial region (the thoracic fraction), where the mucociliary escalator would promote removal via swallowing (OECD, 2009). In most cases, the nasal route is a more efficient particle filter than the oral, especially at low and moderate flow rates. In general, the inhalable fraction is defined as the proportion of airborne particles less than or equal to 100 µm in aerodynamic diameter that can deposit anywhere within the respiratory tract, and the proportion of airborne particles less than 10 µm in aerodynamic diameter that can deposit in the deep lung is defined as the respirable fraction (European Committee for Standardisation (CEN, 1993); this fraction should be considered when calculating inhalation exposure (Schwarz and Koch, 2017).

The Danish Environmental Protection Agency has reported a general assumption that 1% of a face powder is respirable (Danish EPA, 2015). However, as with spray products, particle size is very much triggered by the individual formulation due to the physical chemical properties of its ingredients and their quantities in the product. For example, micronised components could become agglomerated and therefore significantly larger (Cosmetic Ingredient review (CIR, 2012; Farber, 1972).

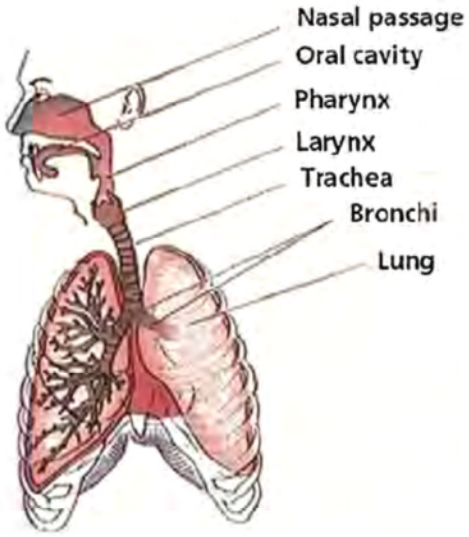
The cosmetics industry has, for many years, utilized several techniques for determining particle size and quantifying the respirable fraction of an airborne material, including laser diffraction, time of flight spectroscopy or the use of so called cascade impactors (FEA, 2009), to satisfy the requirements of the Aerosol Dispensers Directive 75/324/EEC (Aerosol Dispensers Directive (ADD, 1975) and the EU Cosmetics Regulation (EC) No. 1223/2009 (EU, 2009).

The pragmatic laser diffraction technique, using volume based measurements rather than the preferred mass based measurements, is often used especially during product development to get a rough understanding on spray composition regarding particle sizing at initial aerosol generation. By comparison of laser diffraction pictures, the impact of specific formulations and spray can characteristics can be visualized.

As mentioned above, maturation (size reduction by solvent evaporation) of particle size does not typically occur with airborne powders (Schwarz and Koch, 2017), but gravity of bigger particles (agglomerates/aggregates) is still a relevant factor clearing up the atmosphere and minimizing any inhalation exposure (Byrne, 1998; Lu and Howarth, 1995). Consequently, as a first rough estimate, the particle sizes may be simply determined using a graticule measurement of a sample of powder under a microscope but understanding that this technique has a high degree of uncertainty (Allen, 2003; Nazarenko et al., 2012a).

To obtain the most realistic particle size distribution data, time of flight spectroscopy could be employed to measure the particle size distribution over time during a suitably designed simulated use evaluation study (Carthew et al., 2002). In these studies, the consumer use scenario is closely mimicked, such that the aerosol or atmosphere generated from the cosmetic product is as realistic as possible to that might be experienced by the consumer. The spectrometer measures number concentration and size distribution of particles in the room air in which the cosmetic product was released for a period that encompasses the duration of generation and a subsequent interval equivalent to the consumer performing another task in the same room and then the data can be converted to particle mass concentration. However, while simulated testing scenarios have some limitations with regards to fully representing the conditions a consumer is exposed to when applying a cosmetic product, they are designed to be as realistic as possible and they are useful for gaining a better understanding of particle size fractioning.

Quite different to the former methods is the use of cascade impactors, where a kind of battery of filters with different net sizes is combined and exposed to airborne samples. Particles are specifically collected on these filters (impacted) by a standardized vacuum depending on their aerodynamic diameter. The weight increase (mass of impacted particles) of the individual filters will give information on the specific fractioning of the dust probe following the definitions of Fig. 3. The sampling and analysis of particle fractioning with cascade impactors is typically performed at intended use conditions of the final cosmetic product, applying some standard parameters as detailed in the



Nasopharyngeal region

Deposition:	impaction, diffusion
Clearance:	mucociliary, sneezing/blowing
Pathology:	inflammation, cancer, ulceration
Targeted by:	>30 µm particles highly reactive, water soluble gas, "inhalable fraction"

Tracheobronchial region

Deposition:	impaction, sedimentation, diffusion
Clearance:	mucociliary, coughing
Pathology:	obstruction, irritation, cancer, ulceration
Targeted by:	10-30 µm particles, 200 µm fibres, "thoracic fraction"

Pulmonary region (parenchyma)

Deposition:	sedimentation, diffusion
Clearance:	phagocytosis, solubilisation, interstitial
Pathology:	inflammation, fibrosis, cancer, oedema, emphysema
Targeted by:	<10 µm particles, 10-12 µm fibres less reactive/water soluble gas, "respirable fraction"

Fig. 3. The human respiratory tract (Steiling et al., 2014).

following chapters of this article.

7. Exposure assessment

If a cosmetic powder has the potential to generate a dust cloud or atmosphere with respirable particles during handling or application, a proper risk assessment requires determination of consumer exposure in a tiered approach, as shown in Fig. 4.

Inhalation exposure to pressed powders is considered negligible as it is considered unlikely that a significant dust cloud or atmosphere could be formed during the typical application method and due to the low usage amount (approximately 10 mg/application) (Scientific Committee on Consumer Safety (SCCS, 2016).

For loose powders, even with low amounts per application, the principles of the tiered approach to inhalation exposure assessment shown in Fig. 4 may be useful.

7.1. Exposure modeling

During the last decade, several *in silico* models have been developed to calculate the exposure via inhalation based on defined physical chemical parameters of the product/substance and some standardized descriptors of the exposure scenario. Table 1 gives an overview of to day's most widely used computer exposure models and their typical applicability arena for a broad range of spray products even beyond cosmetic products. Most of the mentioned computer models are pub licly available and use some crucial inhalation parameters like breathing rate, room volume, quantity of sprayed product and time of residence in addition to the model specific characteristics to get a trustful estimate of inhalation exposure of an individual in a room with airborne particles.

Although, these exposure models, such as the "RIFM 2 box indoor air dispersion model" (Research Institute for Fragrance Materials (RIFM, 2010), have been designed for spray products, they are gen erally applicable for loose powders. With their typical default para meters, the outcome may be useful even for cosmetic powders as a conservative initial assessment. As sedimentation, an important factor for airborne particles, is not taken into account in these models, the calculated exposure becomes worst case. Specific parameters (e.g. duration of application, amount used, residence time in the bathroom) would have to be adapted to the habitual use of the cosmetic powder. Table 2 details some specific data available in the literature for cos metic powder products that may be suitable for populating exposure models. Additional investigation may be needed to fit the available exposure models for solids beyond using the mentioned powder specific data. If refinement is required to achieve a realistic safety assessment, the tiered approach of exposure assessment has to be followed.

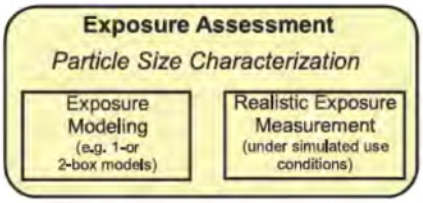


Fig. 4. Step by step approach for the exposure assessment (Steiling et al., 2014).

7.2. Exposure measurement

If having attained a conservative initial assessment using the men tioned modelling approaches and further refinement of the consumer exposure is required to achieve a satisfactory safety assessment, then the next approach for loose powders may be the measurement of powder exposure under simulated use conditions (Carthew et al., 2002). In such tests, the product is applied to a mannequin in the same manner as a consumer would use it. The mannequin is equipped with an simulated upper respiratory tract connected to a time of flight spec trometer and therefore realistic data on inhalable (for potential de position in the whole respiratory tract) and respirable (for potential deposition in the alveolar region of the lung) particle fractions can be obtained by sampling air from the 'breathing zone' of the mannequin. An estimate of consumer exposure is then determined by applying realistic in use conditions, such as human physiological breathing rate.

In cases where a technically equipped mannequin is unavailable, the use of multistage cascade impactors in standardized exposure cubes may function as sufficient surrogates. During such tests, the powdered product is sprayed, puffed or even scattered, in cases where products are tested with such intended application, into a dimentionally stan dardized room ensuring a homogenious distribution of the airborne particles. Well positioned samplers (multistage cascade impactors) en sure the collection of particles in fractions as defined in Fig. 3 (inhal able, thoracic and respirable) (Schwarz and Koch, 2017).

For all these studies, it is important to fully understand the

Table 1
Consumer exposure models for spray products (BAMA: British Aerosol Manufactures Association, BAuA: German Bundesamt für Arbeitsschutz und Arbeitsmedizin, FEA: European Aerosol Federation (Bascompta et al., 2013), RIFM: Research Institute of Fragrance Materials, RIVM: Dutch Rijksinstituut voor Volksgezondheid en Milieu).

Exposure model	Products for which the model is useful
BAMA/FEA Indoor Air model (one-box)	Products sprayed into the air (e.g. air freshener) Products sprayed onto a horizontal surface (e.g. carpet cleaner)
RIVM ConsExpo 4.1 model (one-box) (RIVM, 2016)	Products sprayed into the air (e.g. air freshener) Products sprayed at the body (e.g. cosmetic products) Products sprayed at a vertical surface (e.g. paints) Products sprayed on to a horizontal surface (e.g. carpet cleaner)
BAuA SprayExpo 2.0 model (one-box)	Products sprayed into the air (e.g. air freshener) Products sprayed towards a surface (e.g. paints)
RIFM 2-Box Indoor Air Dispersion model (two-box)	Products sprayed into the air (e.g. air freshener) Products sprayed at the body (e.g. cosmetic products) Products that are combustible (candles) Products that are passive or heated diffusers
RIFM Computational-Fluid-Dynamics (CFD) and Multiple Path Particle Deposition (MPPD) model	Products sprayed into the air (e.g. air freshener) Products sprayed at the body (e.g. cosmetic products) Products sprayed at a vertical surface (e.g. paints) Products sprayed on to a horizontal surface (e.g. carpet cleaner)

Table 2
Literature examples of use frequency, usage amounts and respirable fraction for cosmetic powders.

Product type	Parameter	Input mean values	Reference
Dry shampoo	Frequency/day	0.23 (pregnant women)	Ficheux et al., 2015
Eye shadow		0.72	
Compact powder foundation		0.71	
Loose powder foundation		0.74	
Blush		0.73	
Dry shampoo	Amount/ application	2.4 g	Ficheux et al., 2016
Eye shadow		9.1 mg	
Compact powder foundation		59.6 mg	
Loose powder foundation		73.1 mg	
Blush		13.1 mg	
Eye shadow	Frequency/day	0.40 to 0.78	Cosmetic, Toiletry and Fragrance Association (CTFA, 1983
Blusher and rouge		0.55 to 1.24	
Face powder		0.33 to 0.67	
Blusher and rouge	Amount/ application	11 mg	Loretz et al., 2008
Face powder		85 mg	
Eye shadow	Frequency/day	1.2	
Eye shadow	Amount/ application	30 mg	Danish EPA, 2015
Face powder	Respirable fraction	1%	
Baby powder	Amount/ application	107 mg	Moon et al., 2011

consumer use scenario (method and duration of application, amount used per application and duration of stay in the room, which defines the period of sampling, room volume, air exchange in the room) so that a study protocol can be accordingly designed or exposure data with measured values can be adequately calculated. Typical output parameters for these studies include inhalable particle number concentration (particles/cm^3), inhalable/respirable concentration (μg/m^3), particle size distribution (fractioning) and application rate (g/application).

Nazarenko et al. (2012a) reported their version of a simulated use study for cosmetic powders applied to the face of a mannequin head using brushes or pads, while sampling the airborne powder through ports situated in its nostrils. We briefly describe their work so that the safety assessor can better understand the experimental set up required to conduct such a study and to provide some indication of the potential data outputs from this type of study. The safety assessor will also be able to start to think about some of the considerations needed to ensure a relevant exposure scenario is achieved while conducting these types of studies.

Nazarenko et al. (2012a) conducted air sampling for a three minute application period, at a total flow rate of 11 litres/minute (a conservative flow rate equivalent to an 18 60 year old female undergoing light exercise (Yang et al., 2008; US EPA, 2011)), using a scanning mobility particle sizer (SMPS) and a time of flight aerodynamic particle sizer (APS) in parallel. These sampling devices provided airborne particle concentrations and size distributions in the range between 14.1 nm and 20 μm (Fig. 5). From a consumer exposure scenario point of view, the application period of three minutes is a conservative duration, however the SMPS required this interval to scan the entire particle size range.

Nazarenko et al. (2012b) used the data generated by Nazarenko et al. (2012a) to calculate various particle size fractions inhaled and deposited in different parts of the respiratory tract. Inhaled and deposited dose was determined using a bodyweight of 60 kg and an assumed exposure time of one minute (an exposure period that may be more akin to the consumer use scenario). Under the simulated use testing conditions, the highest inhaled particle mass (equivalent to 30 μg/kg bodyweight/application) was found in the fraction 2.5 10 μm, with more than 85% of all inhalable mass deposited in the head region (highest total deposited dose of 32 μg/kg bodyweight/ application), while deposition was less than 10% in the alveolar region (equivalent to approximately 2 μg/kg bodyweight/application). Interestingly, this Nazarenko alveolar deposition fraction is a significant reduction of the 20% alveolar deposition fraction reported for humans by Lippmann (1977) and modelled, using a multiple path particle dosimetry model based on human lung morphology, by Asgharian et al. (2004).

Although this work may not cover all conditions required for a realistic consumer use exposure scenario, e.g. it was just a mannequin head in a small box rather than a full sized mannequin in a small bedroom/bathroom, it does cover some of the key parameters for such a method and it also provides some useful insights into the inhalable and deposited fractions that might be expected from consumer use of a cosmetic powder. For example, it suggests that large particle sizes predominate and that because of this, upper respiratory tract exposure, possibly associated with local effects to surfaces, may be more important than deep lung exposure, where only small amounts of powder deposit.

8. Discussion

In compliance with the EU Cosmetic Regulation all cosmetic

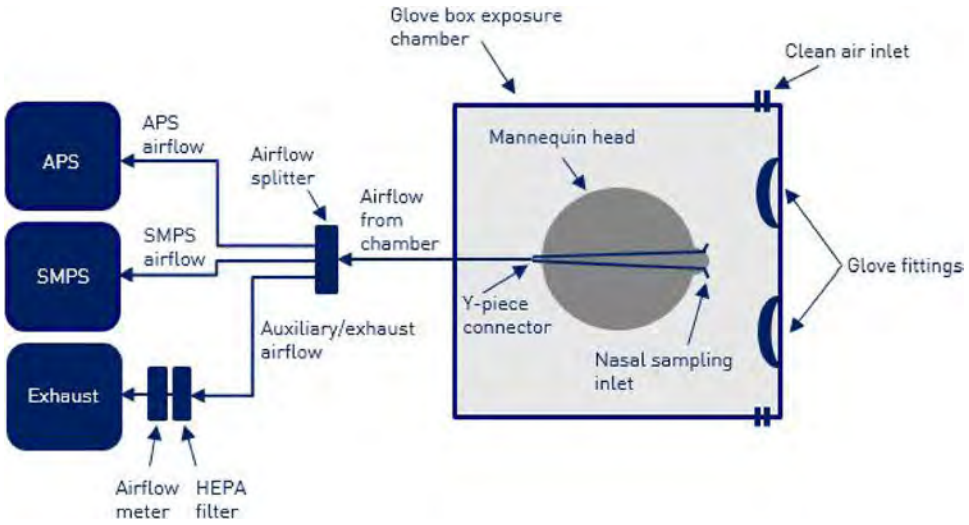


Fig. 5. Diagram of experimental setup for simulated use of a cosmetic powderand measurement of aerosol generated during application; HEPA filter - high efficiency particulate air filter, APS - aerosol particle sizer, SMPS - scanning mobility particle sizer (Nazarenko et al., 2012a).

products including powders must be assessed for their safety taking into account all possible routes of consumer exposure. Cosmetic powders currently in use seem to generate no relevant amounts of inhalable/respirable particles, but have to be evaluated for unintended inhalation exposure in addition to dermal/oral exposure. Both inhalable and respirable particle fractions are defined in Fig. 3, e.g. respirable particles are those with an aerodynamic diameter less than 10 µm that can reach the alveolar region of the lung. Therefore, specific parameters such as the physical format of the cosmetic product, the product container and the application method determine the character of airborne particles when used.

The tiered approach for inhalation exposure assessment, as well as the safety assessment of cosmetic powders, follows the general principles defined for liquid spray products (Steiling et al., 2014). However, in light of the fundamental differences between powders and sprays, additional aspects have been identified which allow certain product types to be safely removed from the inhalation exposure assessment process at an earlier stage, thus reducing the burdon on the safety assessor.

There are two types of powders (compact and loose) with their specific characteristics. Given that compact powders are generally not expected to become airborne at relevant amounts during application, due to the compressed format, method of application and low usage amounts, exposure by inhalation is not expected. Therefore, no specific safety assessment for inhalation exposure is needed for this type of product.

Regarding loose powders, where the particle cohesion of compact powders is lacking, there is the potential for a dust cloud or atmosphere to be formed during handling or use, so the safety assessor should anticipate the potential for inhalation exposure. If the powder is also able to generate respirable particles, consumer exposure by inhalation could be a realistic concern and the safety assessor should follow the different steps and recommendations detailed in this paper. If the product does not generate respirable particles, systemic effects by alveolar uptake at realistically low exposure are unlikely.

Due to the influence of inhalation exposure by several parameters, the tiered approach to inhalation exposure assessment is always recommended.

A calculation (*in silico*) model can be used and implemented with specific parameters (e.g. duration of application, product usage amount and residence time in the bathroom) adapted to the scenario for cosmetic powder applications. However, the safety assessor will need to be mindful that these models are primarily designed for spray products, and even with adaptations for powders the outputs, although useful, will be conservative.

If the application of an *in silico* model demonstrates the safety of the powder at realistic exposure conditions, no further work is needed. However, in some cases, where the model output is very conservative, further refinement may be needed. Finally, exposure measurement under simulated use conditions will help with refining the safety assessment. Realistic consumer exposure measurements will provide a clear insight into the inhalable and respirable fractions that might be expected. Based on the exposure measurements performed with cosmetic aerosols like antiperspirants (Steiling et al., 2012), the exposure is generally many times lower compared to the amount calculated with the *in silico* models. Thus, a safety assessor can expect that unintentional exposure by inhalation during the application of cosmetic powders will also be very low to negligible.

9. Conclusion

The described common principles of exposure assessment are suitable for the safety assessment of cosmetic powders, based on the classical elements of safety assessment. A robust understanding of the special characteristics of powders is key to this approach. Overall, the knowledge of the external, systemic and in particular respiratory tract exposure of potential toxicities and their doses is necessary for the determination of safe exposure levels. This paper is intended to provide basic elements of a tiered safety assessment approach for cosmetic powder formulations in order to increase transparency for regulators and reliability of results to the benefit of the consumer. It provides a recommendation to use these tools in the sense of a Weight of Evidence Approach when conducting the safety assessment.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxlet.2018.08.011.

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